Do seagrasses feel the heat?

Assessing the potential for microevolutionary change in a marine ecosystem engineering plant in response to global warming

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"Knowing trees, I understand the meaning of patience.
Knowing grass, I can appreciate persistence."

Hal Borland, *Countryman: A Summary of Belief*

Contents

Summary

A central question in ecology is how organisms react to changing environmental conditions induced by global climate change. This is particularly important for ecosystem engineering species, as the fate of whole ecosystems is depending upon their performance and survival. In coastal marine habitats, seagrasses are of outstanding importance as ecosystem builders. Eelgrass, the study species of this thesis, is the most widespread and locally abundant seagrass along soft-sediment coasts of the northern hemisphere.

In this thesis I assessed variation among and within eelgrass populations in response to heat stress. I conducted heat stress experiments in a “common stress garden”, simulating a summer heat wave of three weeks followed by a recovery phase. I measured various physiological parameters and assessed the expression profile of selected heat stress associated genes with qPCR as well as the whole transcriptome with next generation sequencing using eelgrass with differing thermal history (a southern population from the Mediterranean Sea and northern populations from the Kattegat and Limfjord, Baltic Sea). To assess variation within populations, I used genotypes originating from a Baltic population.

I found that different genotypes showed varying growth rates in control and heat treatment at acute heat stress, but that all populations lost shoots in response to the heat wave, irrespective of their thermal pre-adaptation. While populations diverged in their expression profiles of selected heat stress associated genes already at the onset of heat stress, subsequent global transcription profiling revealed that those effects were of relatively minor importance compared to massive differences in gene expression during the recovery phase between two of the populations. This is in line with findings on the genotype level within one population which showed differences in the expression profiles of selected stress-associated genes between replicated individuals only in the recovery phase.

This thesis provides a basis for investigating the potential for microevolution of eelgrass populations in the face of global climate change. Both, cold- as well as warm adapted eelgrass populations responded to heat stress with shoot reduction, a finding that is in line with worldwide records of seagrass decline. On the other hand, there is considerable variation for heat stress-related gene expression within populations, a trait that is likely to be important under global change. As this variation

among genotypes is the prerequisite for natural selection and adaptation, populations may succeed to persist.

Deutsche Zusammenfassung

Eine zentrale Frage der Ökologie ist, wie Organismen auf die durch den globalen Klimawandel veränderten Umweltbedingungen reagieren. Besonders wichtig ist dies für Arten, die die Funktion von ‘Ökosystem-Ingenieuren’ übernehmen, da das Schicksal ganzer Ökosysteme von ihren Reaktionen und ihrem Überleben abhängt. Diese Arbeit behandelt das Große Seegras (*Zostera marina*). Es ist die häufigste und am weitesten verbreitete Seegrasart auf Weichböden der nördlichen Hemisphäre.

Ich untersuchte in dieser Arbeit Unterschiede in der Antwort auf Hitzestress zwischen und innerhalb einzelner *Z. marina* Populationen. Hierzu führte ich Hitzestress-Experimente in einem ‘common stress garden‘ durch, in denen ich eine dreiwöchige Sommerhitzewelle mit anschließender Erholungsphase simulierte. Ich bestimmte sowohl an verschiedenen Populationen aus unterschiedlichen Temperaturregimen (südliche Populationen aus dem Mittelmeer und nördliche Populationen aus Kattegat und Limfjord, Ostsee) als auch an verschiedenen Genotypen einer Ostseepopulation mehrere physiologische Parameter, Expressionsprofile ausgewählter Stressgene mittels qPCR sowie das gesamte Transkriptionsprofil mittels Next- Generation- Sequenzierungen (NGS).

Es zeigte sich einerseits, dass sich verschiedene Genotypen in ihren Wachstumsraten unter Versuchs- und Kontrollbedingungen bei akutem Hitzestress unterscheiden. Andererseits reagierten alle Populationen mit Sproßverlust auf die Hitzewelle, unabhängig von ihrer Anpassung an die jeweils im Feld vorherrschenden Temperaturbedingungen. Obwohl Populationen sich im Expressionsprofil ausgewählter Stressgene schon zu Beginn der Hitzewelle unterschieden, zeigte sich, dass diese Effekte relativ unbedeutend waren im Vergleich zu den starken Unterschieden, die im gesamten Transkriptionsprofil (mittels NGS) zwischen zwei der Populationen während der Erholungsphase festgestellt werden konnten. Dies stimmt mit Ergebnissen auf ‚Genotyp‘- Ebene überein, die zeigten, dass Genotypen einer Population sich im Expressionsprofil ausgewählter Stressgene ebenfalls erst in der Erholungsphase nach der simulierten Hitzewelle unterschieden.

Diese Arbeit bietet eine Grundlage zur Untersuchung des Potentials zur Mikroevolution für Populationen des Großen Seegrases angesichts des globalen Klimawandels. Sowohl kalt- als auch warm adaptierte *Z. marina* Populationen reagierten auf Hitzestress mit Sprossverlust, ein Ergebnis, das zu weltweiten

Berichten über den Rückgang der Seegrasbestände passt. Andererseits variieren unterschiedliche Genotypen in ihrer Genexpression unter Hitzestress, einem Merkmal, das unter durch den Klimawandel veränderten Umweltbedingungen wichtig werden kann. Da Variation zwischen Individuen die Basis für natürliche Selektion und Adaptation darstellt, könnte dies den Fortbestand der Populationen des Großen Seegrases ermöglichen.

Introduction

Seagrass ecosystems in the light of global climate change

One key question in evolutionary ecology research is how populations, species and communities will react to the changes induced by global climate change (Lubchenco 1998). These reactions may be particularly important in ecosystem engineering species, as they form, structure and sustain entire ecological communities (Jones et al. 1994). Coastal marine habitats in tropical regions for instance are mainly engineered by mangroves and corals. In temperate ecosystems, salt marshes, kelp beds and seagrass meadows hold a key regulatory role (Orth et al. 2006).

Seagrasses play a major role in the balance of coastal ecosystems. Their meadows provide habitat and food for many marine invertebrate and vertebrate species. They can positively influence other marine ecosystems (reviewed in Heck et al. 2008), for example by contributing to the flotsam by detached leaves that are washed ashore (Hemminga & Duarte 2000). Seagrasses can alter currents with their leaves and stabilize sediments through their rhizome-root-system (Orth et al. 2006), a feature only higher plants possess. This allows them to literally gain ground in sandy sediments, an ecological niche, which algae, lacking a root system, are not able to occupy. In addition, they structure their physical and chemical environment. Seagrasses are important carbon sinks and supply the oceans with nitrogen, phosphor and organic carbon (Duarte et al. 2005; McGlathery et al. 2007). Because they provide essential ecological services to the marine habitat, seagrasses have been rated among the most valuable ecosystems worldwide in economic terms (Costanza et al. 1997).

There is now mounting evidence that seagrass meadows are globally declining (Short & Wyllie-Echeverria 1996), with documented losses that have increased by a factor of 10 over the past 40 years (Orth et al. 2006). Almost one third of the seagrass area identified in the first quantitative record has disappeared and area expansions fall more than 10-fold behind area reductions with loss rates having reached a median of 7 % per year (Waycott et al. 2009). The mean overall seagrass loss equals the reductions in coral reefs and mangrove forests and even exceeds those of tropical forests (Waycott et al. 2009). Nearly a quarter of all seagrass species have been classified as threatened or near threatened under the Categories and Criteria of the International Union for the Conservation of Nature (Short et al. 2011), leading to serious conservation concern.

Various anthropogenic and natural stressors have been identified to drive the decline of seagrasses and range from eutrophication to sea-level rise, mechanical disturbance, hydrodynamic changes, diseases (see below) and extreme climatic events, individually or in combination, (Hemminga & Duarte 2000). A synthesis of representative case studies showed high temperature events to have a major impact on seagrass loss especially in temperate regions (Orth et al. 2006), which is also supported by a model of climate change effects on seagrass stability and resilience (Carr et al. 2012).

Eelgrass: an ideal study system for thermal adaptation

Generally, organisms can respond to changing environmental conditions either by migrating to more benign environments, or by phenotypic and genetic adaptation to the new environmental stressor (Jackson & Overpeck 2000). Empirical evidence suggests migration in contrast to adaptation to play a key role in species' response to global climate change (reviewed in Parmesan 2006). On the other hand, populations have often been shown to be phenotypically and genetically differentiated with respect to climate, indicating that climate may after all impose strong selective pressure (reviewed in Jump & Penuelas 2005). Additionally, many studies across a wide range of taxa have identified adaptive change to be faster than previously thought (Hendry et al. 2008). In summary, more empirical evidence is needed on the adaptive response of natural plant populations to global climate change (e.g. Hoffmann & Sgro 2011; Jump & Penuelas 2005).

An integrative approach, combining genomic approaches like gene expression studies with ecological research in the field, can potentially provide us with the necessary information to understand the evolutionary processes and mechanisms that enable populations to adapt to multifaceted selection pressures generated by global climate change (Hofmann et al. 2005). Although variation in gene expression within and among populations has been identified to be of adaptive importance in response to global warming (Whitehead & Crawford 2006b), we still have little understanding of the reasons that lead to the conservation or loss of genetic diversity in traits affected by global climate change (Jump & Penuelas 2005; Rice & Emery 2003).

In contrast to terrestrial systems (Chown & Gaston 2008; Chown et al. 2004), only few studies exist on thermal adaptation in marine systems and except for corals, a relatively well-studied organism (e.g. Bellantuono et al. 2012; Rodriguez-Lanetty et al.

2009) these mostly deal with ectothermic animals (e.g. Fangue et al. 2006; Osovitz & Hofmann 2005; Sorte & Hofmann 2005; Stillman & Tagmount 2009; Whitehead & Crawford 2006a). Yet, it may seem particularly important to assess the adaptive potential of ecosystem engineering species of also temperate regions to global climate change since their performance and survival can determine the fate of whole ecosystems (Ellison et al. 2005; Jones et al. 1994).

Eelgrass (*Zostera marina*) is the dominant seagrass in the northern hemisphere, but is distributed globally along the coasts of the Pacific as well as the Atlantic, occurring in subarctic as well as in subtropic regions (den Hartog 1970). *Z. marina* is a clonal plant that constantly builds new vegetative ramets out of a rhizome. Once a year it reproduces sexually by producing generative shoots that detach after flowering. Containing pollinated seeds, these floating shoots provide an important means of dispersal. The vegetative shoots can detach from the feeding rhizome resulting in unconnected, but genetically identical ramets, which may become exposed to different environments.

Molecular tools are evolving quickly for *Z. marina*. An EST database has already been established; the installation of qPCR assays and next generation sequencing studies have added to the picture and whole genome sequences are currently processed. Together with this rich library of genetic information the possibility for clonal replication facilitates exposing units of equal genetic identity to different treatments in experiments and makes *Z. marina* an ideal study organisms for evolutionary ecology, particulary in ecosystem genetics research (Whitham et al. 2006). Additionally, its occurrence over a vast thermal range facilitates elucidating the genetic basis of thermal adaptation by comparing differences in local gene expression patterns (Oleksiak et al. 2002; Whitehead & Crawford 2006a).

Genes underlying plant stress tolerance

Because migration is not an option for sessile organisms as an immediate reaction to stress, morphological, physiological, biochemical and molecular reactions to abiotic stress are crucial adaptations for plants (Wang et al. 2003). Acute stress activates signaling processing and transcription controls, which trigger stress-responsive mechanisms aimed to restore cellular homeostasis. Inadequate response at any of these

steps may lead to irreparable disturbance of cellular homeostasis, a collapse of cellular organization and ultimately result in cell death (Wang et al. 2003).

Molecular chaperones are part of various cellular processes regulating protein folding, assembly, translocation and degradation under normal conditions. Additionally, they are induced by changes in various stressors such as increases in temperature (Vierling 1991), playing an essential role for cellular homeostasis under stress, as they assist in protein refolding and thus stabilize proteins and membranes. One major group among the molecular chaperones is built by the heat-shock proteins (Hsps) that were originally identified to be involved in the heat stress response (Lindquist 1986). Hsps include five different families that were classified and named according to their molecular weight, namely the Hsp70 family, the Chaperonins including the Hsp60 family, the Hsp90 family, the Hsp100 family and the small Hsp (sHsp) family (Wang et al. 2004) Although Hsps are ubiquitous in the cell (Boston et al. 1996), the different classes have very specific functions for homeostasis (Wang et al. 2004). The induction of Hsps is a conserved response to heat stress observed in a wide range of organisms (Vierling 1991). Their expression level has been identified to be under selection particularly in connection with relatively rare, unexpected extreme stress events like heat waves (Sorensen et al. 2003).

In terrestrial plants that face drastic temperature changes during the course of a day, sHsps have an unusual abundance and diversity (Vierling 1991) compared to other organisms, and are thus hypothesized to have a special importance in the plant heat stress response (Wang et al. 2004). In contrast to reactions of terrestrial plants to abiotic stresses, which have achieved considerable attention especially in the context of agriculture (e.g. Wahid et al. 2007), the stress response of marine macrophytes including seagrasses remains largely unexplored.

Seagrasses are the only higher plants in the marine realm that evolved from monocotyledonous flowering plants about 100 million years ago (Les et al. 1997). Having reinvaded marine coastal habitats from freshwater environments (Les et al. 1997), they still possess typical features of their terrestrial ancestors such as an aerenchym, a rhizome-root-system, vegetative shoots with short stem and long ribbon-like leaves, and generative shoots with inflorescences. Seagrasses also show unique reproductive adaptations to a life submerged in water including subaqueous flowering, hydrophilous pollination (all species but Enhalus acoroides) and seeding (Cox 1993), as well as physiological and morphological adaptations like epidermal chloroplasts and internal gas

transport (den Hartog 1970; Les et al. 1997). These adaptations to the aquatic environment also make seagrasses particularly interesting as a study system for adaptation including the determination of functional gene divergence driven by the new selection pressure (Wissler et al. 2009).

One major difference between terrestrial and aquatic environment is the thermal condition of the medium (Feder & Hofmann 1999). On the one hand temperature changes more slowly in water than in air due to the high specific heat and thermal conductivity of water, leading to a relatively stable thermal environment (Reusch & Wood 2007). On the other hand, once a critical temperature is reached, it lasts much longer than on land, leading to extreme stress in the affected organisms (Reusch & Wood 2007). Particularly affected are sessile organisms occupying the intertidal zone or shallow waters like tidal ponds and creeks (Feder & Hofmann 1999). Among these organisms, corals and their reactions to thermal stress have a quite long history of research (e.g. Brown & Howard 1985) with the death of their endosymbionts leading to coral bleaching even with very subtle temperature changes of 1 to 2 degrees. In comparison, the reaction to thermal stress in seagrasses is poorly understood.

Seagrasses from a different perspective: The pathogen *Labyrinthula*

Seagrasses are not only threatened by anthropogenic climate change, they also have been heavily impacted by marine diseases. The eelgrass wasting disease in the 1930s led to a pandemic decline of over 90% of the eelgrass populations along the Atlantic coasts of Europe and Northern America within a few years (reviewed in Muehlstein 1989). Due to the severity of this outbreak, eelgrass wasting disease is regarded as a one of the most critical events in seagrass population biology (Milne & Milne 1951) and on the level of marine ecosystems, is considered a major epidemic disease (Muehlstein 1989).

The typical symptoms for infection with the wasting disease are small dark spots and streaks on eelgrass leaves, that increase in size, leading to leaf loss and ultimately to the death of the plant. Various abiotic and biotic causes for these symptoms have been hypothesized involving changes in local environmental conditions like temperature, salinity, precipitation, pollution and solar cycles, as well as infection by bacteria (reviewed in Muehlstein 1989). Using Koch's postulates, Muehlstein et al. (1991) identified *Labyrinthula zosterae* to be the causative agent. It mainly develops in *Z.*

marina leaf parenchyma cells and causes the symptoms of the wasting disease by damaging chloroplasts leading to reduced photosynthetic activity (Raghukumar 2002; Renn 1936).

L. zosterae is a protist within the group of stramenopiles that belongs to the class Labyrinthulomycetes (Tsui et al. 2009). They are commonly associated with marine macroalgae and seagrasses (Bockelmann et al. 2012; Vergeer& den Hartog 1994) and have more recently also been identified to cause severe outbreaks of the wasting disease in terrestrial grasses (Douhan et al. 2009; Hyder et al. 2010 ; Olsen 2007).

Although *L. zosterae* has been identified as causative agent, the epidemiology of the wasting disease and the role of environmental factors influencing infection largely remain unresolved with contradicting evidence particularly on whether higher salinities trigger infection (e.g. Bockelmann et al. 2012; McKone & Tanner 2009). In the light of global climate change being linked with a prospected increase of marine diseases (Harvell et al. 2002) the picture becomes increasingly complex. Global warming is anticipated to lead to temperatures optimal for the growth and reproduction of *L. zostera* (McKone & Tanner 2009) in the temperate waters of the Baltic and North Sea (Bockelmann et al. 2012), which very likely adds another stressor to the network of factors leading to increased conservation concern for seagrass.

Thesis outline

This thesis contains four chapters, each of which is organized in manuscript form and contains the sections abstract, introduction, material and methods, results and discussion. Here I briefly introduce the research questions of the single chapters and explain the reasons for the respective approaches.

Chapter I

In the first chapter I developed qPCR assays of heat stress related candidate genes and assessed differences in gene expression between eelgrass shoots from populations with different thermal pre-adaptations as a response to a simulated heat wave. By the time this study was conducted, the EST-database Dr. Zompo was only just installed by Reusch and co-workers. This database was an invaluable source of information, allowing a focused search for potential target genes and the design of primers that cross exon-exon boundaries in seagrass. The qPCR assays developed here were a prerequisite to answer the main question: Is the different thermal history of eelgrass shoots from southern populations (Mediterranean Sea) and eelgrass shoots from northern populations (Baltic Sea) reflected in differential physiological performance and target gene expression when exposed to an experimental heat wave?

Chapter II

The second chapter is closely connected to chapter I, as identical RNA samples were processed in the two chapters. Because techniques advance quickly in the field of molecular research, we were able to complement the results from Chapter I with a large scale transcription profiling using next generation sequencing. The main questions in this chapter were, first, if there are differences in the whole transcriptome of two populations with contrasting thermal pre-adaptation as a response to an experimental heat wave and second, which genes and functional gene groups are important in explaining that variation.

Chapter III

In the third chapter I leave the subject of gene expression differences between populations and assess variation on a smaller scale – I investigate differences in gene expression among different genotypes of one single populations. Using three indicator genes for heat stress identified in Chaper I and II, I assessed differences in physiological traits and gene expression during the course of an experimental heat wave between 5 different genotypes of a northern Baltic Eelgrass population that rarely has experienced heat stress before. The main question was whether there is variation in the heat stress answer among individual genotypes - a prerequisite for microevolution in the face of global warming.

Chapter IV

The forth chapter is a technical description of the development of a qPCR assay for identification and quantification of *Labyrinthula zosterae* cells. The first experimental setup for Chapter I was conducted in 2007. After some days in the mesocosms, eelgrass plants began to show black spots and died shortly afterwards. After three weeks I had lost all shoots. For further experiments to be successful I investigated the reasons for the mass mortality and one of the prime suspects was infection with the seagrass pathogen *Labyrinthula*. Reviewing the literature I found that a fast and precise method of identification and quantification of *Labyrinthula* was lacking. I aimed at providing an identification method as a basic experimental tool – not only for my experiments but as a basis for general research on *Labyrinthula* and host-pathogen interactions in seagrasses.

Chapter I

The AQUATRON in Münster –
a unique seagrass culturing facility

View into a *Zostera marina* experimental patch

Chapter I

Population-specificity of heat stress gene induction in northern and southern eelgrass *Zostera marina* populations under simulated global warming

Abstract

Summer heat waves have already resulted in mortality of coastal communities, including ecologically important seagrass meadows. Gene expression studies from controlled experiments can provide important insight as to how species／genotypes react to extreme events that will increase under global warming. In a common stress garden, we exposed three populations of eelgrass, *Zostera marina*, to extreme sea surface temperatures, simulating the 2003-European heat wave. Populations came from locations widely differing in their thermal regime, two northern European locations [Ebeltoft (Kattegat), Doverodde (Limfjord, Baltic Sea)], and one southern population from Gabicce Mare (Adriatic Sea), allowing to test for population specificity in the response to a realistic heat stress event. Eelgrass survival and growth as well as the expression of 12 stress associated candidate genes were assessed during and after the heat wave. Contrary to expectations, all populations suffered equally from three weeks of heat stress in terms of shoot loss. In contrast, populations markedly differed in multivariate measures of gene expression.

While the gene expression profiles converged to pre-stress values directly after the heat wave, stress correlated genes were upregulated again four weeks later, in line with the observed delay in shoot loss. Target genes had to be selected based on functional knowledge in terrestrial plants, nevertheless, 10 ／ 12 genes were induced relative to the control treatment at least once during the heat wave in the fully marine plant *Z. marina*. This study underlines the importance of realistic stress and recovery scenarios in studying the impact of predicted climate change.

Introduction

How populations, species and communities react to global change is a question of central importance for biological research in the 21st century (Lubchenco 1998). To this end, molecular genetic approaches may provide important new insights. In particular, gene expression profiling can serve as tool to link the genotype with physiology and the phenotype (Hofmann et al. 2005).

Gene expression variation within and among populations is one prime resource for rapid evolutionary change in response to global warming (Michalak et al. 2001; Whitehead & Crawford 2006b; Reusch & Wood 2007). It follows that populations of the same species, living in thermally contrasting environments should reveal the genetic basis of thermal adaptation and tolerance in terms of divergent gene expression (Whitehead & Crawford 2006a). In ecosystem engineering species (sensu Jones et al. 1994), the fate of an entire associated community depends upon the population persistence of a single species. This applies to seagrasses, a group of ecosystem engineering species that provide the foundation of an entire ecosystem of associated plants and animals (e.g. Orth et al. 2006; Hughes et al. 2009b). At the same time, many seagrass based ecosystems are decreasing worldwide (Waycott et al. 2009), prompting the question as to how global warming may exacerbate this decline (e.g. Procaccini et al. 2007). This probably explains why population declines and physiological damages of elevated temperature on *Zostera marina* have recently received considerable attention (e.g. Williams 2001; Greve et al. 2003; Reusch et al. 2005).

The current work focuses on the assessment of fitness associated traits along with quantification of gene expression under experimentally induced heat stress in the dominant seagrass of the northern hemisphere, *Z. marina*. One major contribution of gene expression profiling is a precise prediction of the effects of thermal stress on organisms and the consequence on species' distribution patterns (Helmuth et al. 2002). Because the induction of stress associated genes may take place long before phenotypic effects become apparent, particular stress inducible genes may even serve as early warning indicators (Hoffmann & Daborn 2007). Although experiments on thermal adaptation are prevalent in terrestrial systems (e.g. Chown et al. 2004; Chown & Gaston 2008), information is scarce for marine systems. To date, most studies deal with ectothermic animals (Osovitz & Hofmann 2005; Sorte

& Hofmann 2005; Fangue et al. 2006; Whitehead & Crawford 2006a; Stillman & Tagmount 2009) while for marine ecosystem engineering species, studies are often restricted to corals (e.g. Rodriguez- Lanetty et al. 2009; Voolstra et al. 2009; Desalvo et al. 2010; but see Lago-Leston et al. 2010).

In species with a distribution along a thermal cline, theory predicts that locally adapted populations from southern regions display a higher thermal tolerance than northern ones, thus providing the former population with a potential for genetic rescue against increasing temperatures (Davis & Shaw 2001). This may apply to populations of *Z. marina* within Europe, which grow from the subarctic regions of the White Sea to the subtropical areas of southern Portugal. Such species distributed along a wide-ranging thermal gradient are also ideal to study possible differences in local gene expression patterns (Oleksiak et al. 2002). One major hypothesis of this work was that under experimentally imposed thermal stress, populations from southern origin (Adriatic Sea) should be more tolerant to heat stress than northern populations (Baltic and North Sea). Correlated with their ecological performance, we also expected that northern and southern populations show divergent gene expression patterns. We here coupled phenotypic assessments with gene expression profiling, because the rapid evolution of gene expression has been hypothesized to underlie thermal adaptation in contrasting environments (Michalak et al. 2001). This implies that gene expression is heritable, including an additive genetic variance component (reviewed in Gibson & Weir 2005; Skelly et al. 2009).

To investigate these predictions we assessed a relatively small number of stress associated genes (12) over several time points before, during and after a heat wave. Gene expression profiles were assayed using (real time) quantitative PCR (qPCR), including a long recovery phase of four weeks after the experimental heat wave. Such a target gene approach allows for deeper biological replication and is thus complementary to global transcription profiling that is now underway in many non-model organisms (e.g. Reusch et al. 2008; Rokas & Abbot 2009). For candidate gene selection we systematically targeted molecular chaperones, a group of proteins with a crucial role under stress for protein refolding and in maintaining cellular homeostasis in many organisms (reviewed e.g. in Feder & Hofmann 1999; Wang et al. 2004; Wandinger et al. 2008). Among the chaperones, heat shock proteins (Hsps) are a widespread and conserved group of proteins that are induced under heat stress, typically when the normal range of the thermal environment characteristic for a

species or population is exceeded (reviewed in Feder & Hofmann 1999 for animals; Wang et al. 2004 for plants).

Although they may use the same gene repertoire, marine organisms may react very differently to thermal stress compared to terrestrial organisms, for which the majority of studies thus far have been conducted. Cooling by means of evaporation is not possible in the sea, while temperature conditions within the aqueous medium are locally less heterogenic compared to terrestrial habitats (Feder & Hofmann 1999). Moreover, since water possesses a much higher heat capacity than air (Steele 1985), temperature increases forced by the atmosphere come slower, but persist longer. An additional goal was therefore to assess whether or not the selected candidate genes were associated with the heat stress response in a strictly marine plant, the seagrass *Z. marina*, although the function of these genes was inferred based on terrestrial plants.

Our study builds upon previous genetic and genomic work on seagrasses (reviewed in Procaccini et al. 2007), but proceeds an essential further step by combining the assessment of phenotypic and gene expression data in a realistic stress garden experiment during heat stress and importantly, the recovery period. To this end, we utilized recent EST library resources developed for seagrasses (Reusch et al. 2008; Wissler et al. 2009) that allow for a systematic search of candidate genes related to the stress response.

We compared responses of three *Z. marina* populations originating from habitats with different temperature profiles in response to a simulated realistic heat wave scenario based on data from the Baltic summer heat wave 2003 (Reusch et al. 2005). Our goals were to (i) describe the heat stress response in a marine foundational plant, using a realistic rate, intensity and duration of heat stress; (ii) assess whether the pattern of gene expression in 12 stress associated genes differs among populations from contrasting thermal habitats over the course and recovery period of the heat wave and (iii) test whether *Z. marina* populations from contrasting thermal origins differ in their tolerance to a heat wave—consistent with predictions of local adaptation.

Materials and methods

Study species

Our study species, *Z. marina* (eelgrass) belongs to the seagrasses, a polyphyletic group of about 50 species of angiosperms that returned to the marine environments during the Cretaceous (Les et al. 1997). *Z. marina* reproduces clonally by vegetative growth and sexually via submarine pollination and seeds. This species is the dominant seagrass in temperate coastal waters of the northern hemisphere with a distribution on both, Atlantic and Pacific coasts (den Hartog 1970). Seagrass meadows, including *Z. marina* meadows, are the basis of highly diverse ecosystems as feeding and nursery habitat for various invertebrates and fish species (e.g. Jackson et al. 2001; Dorenbosch et al. 2005) and provide food for waterfowl (Nacken & Reise 2000). While their root and rhizome systems stabilize sediments, the leaf canopy alters the hydrodynamic environment (reviewed in Madsen et al. 2001), and allows suspended particles to sediment (e.g. Terrados & Duarte 2000).

Experimental setup

Z. marina shoots (with attached roots) were collected in two northern European locations, Doverodde (Denmark; North Sea; N56° 43.07’ E08° 28.45’) and Ebeltoft (Denmark; Baltic Sea; N56° 12.50’ E10° 34.65’), and in one southern European location, Gabicce Mare (Italy; Adriatic Sea; N43° 57.97’ E12° 45.86’), during late spring 2008. Sampling was done at ambient water temperatures (12–16 °C) in a water depth of 1.5–3 m. Special care was taken not to disrupt the rhizome connections. Plants were transported to the AQUATRON Münster, a seagrass culturing facility containing 12 mesocosms (101 cm ・120 cm ・86.5 cm) connected in two closed seawater circuits with a flow rate of 1200 L／h.

Shoots were planted between 20 April and 8 May within 48 h after uprooting. Each tank contained 6 boxes of 36.5 cm ・ 26.5 cm area, filled to a height of 10 cm with natural sediment from the Baltic Sea (N54° 24.37’ E10° 11.44’). Two boxes per tank were planted with shoots from each of the three locations. We performed a preliminary study that showed no effect of sediment type (beach sand, sediment from

meadow, beach sand inoculated with meadow sediment) on seagrass growth and survival (see Appendix I-S3).

Previous field collections and microsatellite genotyping revealed that it was sufficient to collect at 5 m intervals along a transect line in each location in order to prevent sampling of identical clones (Bergmann et al., unpublished data). Hence, at each location, 30 leaf shoots (= ramets) were collected within 15 spots of approximately 50 cm in diameter > 5 m distant from each other. Ramets were planted such that each box received plants from seven such sampling spots, which putatively corresponded to seven random genotypes within each box, from a larger sample of 15 genotypes from each population. We later assured via microsatellite analysis that genotypic diversity was indeed maximal in all experimental treatments although populations differed moderately in their genotypic diversity (see Material and methods: genotyping, RNA-extraction and cDNA preparation). Each tank housed 50 periwinkles (*Littorina littorea*) to control epiphytic algae growth.

Two circulating systems filled with artificial seawater (Instant Ocean®) allowed a gradual acclimation of shoots from their local salinity at the time of sampling with an increment of no more than 1 psu ⁄ day. The chosen salinity was within the range of salinities experienced by all three local populations, including Ebeltoft, where at a nearby station (Aarhus bight) salinities attain 30 psu (Fig. I-S2, Appendix; and Pejrup et al. 1996). When reaching 31.5 psu, both flumes were connected and the water temperature was increased from 14 °C (collection temperature) to 19 °C in steps of 0.5 °C⁄ day, followed by an acclimation period of 21 days. Shoots were provided with light at saturation levels with 200 µmol photons ⁄m2 ⁄ s at the surface of the leaves in a 16 h ⁄ 8 h light–dark cycle using two 400 W bulbs per tank (one Philips Master Green Power T 2000K, 745 µmol ⁄ s; one Philips Master HPI-T PLUS 4000K, 532 µmol ⁄ s). Water was fertilized during the heat wave to attain nutrient values in temperate coastal waters of approximately 40 µM N and 3 µM P that are not growth limiting. At the end of the heat wave, the illumination was changed to 15h ⁄9 h and fertilization was stopped to realistically simulate nutrient depletion during summer stagnant conditions.

Mean nutrient concentrations were 34.17 µM ± 1.63 SE for nitrate and nitrite, 1.47 µmol ⁄ L ± 0.62 SE for ammonium, 1.61 µM ± 3.50 SE for silicate and 0.43 µM ± 0.15 SE phosphate. All tanks of control and temperature treatment were exposed to identical modifications of illumination and fertilizations. Thus we conclude that any

observable difference between control and temperature treated plants must arise from the temperature manipulations or an interaction of the temperature manipulations with the illumination / fertilization shift. Either way, both scenarios would reflect a relevant response to heat stress in the natural environment.

Experimental treatments

The experimental treatments were chosen such that for the two northern locations (Doverodde and Ebeltoft), the heat wave constituted an extreme event, while it is regularly experienced each summer by the southern population (Gabicce Mare). Accordingly, seagrass populations in the Adriatic Sea regularly cope with summer temperatures over 26 °C, whereas northern populations (Doverodde and Ebeltoft) never experienced such summer temperatures during the last years (22 and 6 years, for Doverodde and Ebeltoft, respectively, see Fig. I-S1, Appendix I-S2), with the rare exception of the 2003 European heat wave with a return time of >10 000 years (Schär et al. 2004).

Between 4 and 9 June 2008, the temperature in tanks randomly designated to the heat wave treatment was raised from 19 to 26 °C with temperature increments of maximally 1.5 °C/ day, which corresponds to natural rates in Baltic lagoons (Reusch et al. 2005). Upon reaching 26 °C, this temperature was kept for three weeks to resemble the 2003 summer heat wave situation in the Baltic Sea (Reusch et al. 2005). Thereafter, temperature was decreased to 19 °C in steps of max 1.5 °C/ day and then kept constant at 19 °C for another four weeks to test for longer-term effects. Tanks of the control treatment were always kept at 19 °C (Fig. 1). In order to prevent the water chemistry to diverge across treatments, the water was exchanged between the two flumes at a rate of 1200 L / h.

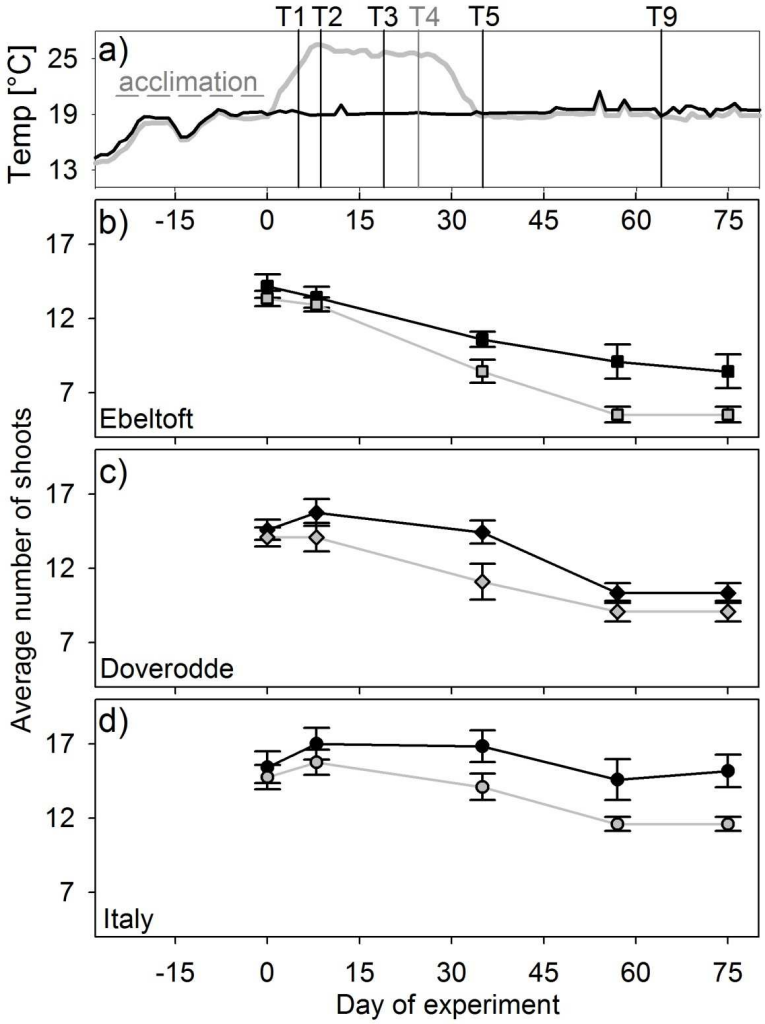

Fig. 1: Time course of temperature and shoot count in *Z. marina* (eelgrass). In panel (a) experimental temperatures are given (grey: heat stress treatment; black: control tanks) while vertical lines indicate days of RNA-sampling (Timepoints T1–T9). Marked in grey are acclimation phase and time point 4, which was omitted from qPCR analyses (see Supporting information on pooling procedure I-S4, Appendix). The spikes in the control at days 12, 49 and 58 correspond to small failures of the cooling of the experimental facility AQUATRON. Panels (b)–(d) give mean number of shoots per box for locations Ebeltoft, Doverodde (northern location) and Gabicce Mare (southern location), respectively.

As fitness correlated traits, we measured leaf growth in weekly intervals, and shoot numbers biweekly. Growth rates of one randomly picked shoot per box were assessed once per week. This was done by measuring the distance from the basal non-growing leaf sheath to the tip of the three youngest leaves before and after a 72 h interval. Growth increments were added and standardized to 24 h. None of the leaf shoots was measured twice throughout the experiment.

Genotyping, RNA extraction and cDNA preparation

In order to quantify neutral population differentiation, samples of 40 shoots per population were genotyped using nine microsatellite loci (Reusch et al. 1999; Reusch 2000). As genotypic diversity has been shown to have an impact on stress resilience in *Z. marina* (Reusch et al. 2005; Ehlers et al. 2008), we verified the clonal diversity among the experimental plants from each location. DNA samples were obtained from each individual shoot at the end of the experiment by cutting off 2 cm of the tip of a leaf. Genotyping was done using standard protocols of four particularly polymorphic microsatellites (AJ009898, 009900, 249305, 249307), with the modification that a direct-PCR approach (Phire Plant Direct PCR Kit, Finnzymes, Finland) was used for PCR amplification. Genotyping and size-separation were performed on an ABI 3130・l capillary sequencer. Clones were assigned to multi-locus genotypes using the P_{sex} approach of Parks & Werth (1993) that calculates the likelihood to obtain a given multi-locus genotype by chance. Population differentiation was calculated as Wright's F_{ST}, estimated according to Weir & Cockerham (1984) with the software Microsatellite Analyser (Dieringer & Schlotterer 2003).

For gene expression, RNA samples were taken between 10 and 12 h to control for circadian gene expression on day 5, 8, 14, 28, 35 and 63 of the experiment (Fig. 1). A 2-cm leaf piece (youngest leaf) was obtained from the same randomly selected plants that had been used for growth rate measures, wiped clean and dipped into liquid N_2 followed by immediate extraction. In a separate analysis, it was found that repeated cutting of leaves increased leaf growth rates (data not shown), indicating compensatory growth, while a single sampling changed growth rates only little. In total, 360 samples (72 samples at each of five time points) were extracted with the Invisorb RNA plant HTS 96 extraction kit (Invitek, Berlin, Germany) following manufacturer's instructions. Samples were disrupted consecutively in batches of 32 and only 300 µL of lysate and binding buffer was used. Reverse transcription was performed with the Quantitect Kit (Qiagen, Hilden, Germany), following standard protocols. For gene expression analysis, we randomly selected five different genotypes from each treatment x population ・ time point combination (n = 12). In order to obtain a qualitative overview of responsive genes and time differences, we utilized a RNA pooling approach by first measuring gene expression in pooled samples

where equal molar contribution was adjusted after RNA quantification (see I-S4, Appendix).

Target gene selection and QPCR

Our goal was to assess differences in the transcriptomic response between warm- and cold-adapted *Z. marina* populations, following a target gene approach. In order to select appropriate genes, 134 putative target genes were selected using the key words 'heat shock' and 'heat stress' in the Dr Zompo database (http://drzompo.uni-muenster.de/; Wissler et al. 2009). The homology search revealing functional information encompassed multiple data bases (Pfam domains, GeneOntology and KEGG). This selection was narrowed down to 34 target sequences, encompassing Hsp encoding genes or other chaperones. These were aligned with putative homologous sequences obtained through BLASTX searches against the *Arabidopsis* Information Resource (TAIR) and the Rice Genome Annotation Project using BioEdit (Hall 1999). Primers were designed with the primer analysis software Primer3 (Rozen & Skaletsky 2000) with a target T_M of ~60 °C (Table S1, Appendix). Forward and reverse primers were placed across exon–exon boundaries to avoid genomic amplification. We were able to establish quantitative real-time PCR assays for 12 genes (Tables 1 and I-S1, Appendix, Molecular Ecology Resources Primer Database IDs 43752–43764). In one case, we found the same Swiss-Prot hit for two different tentative unigenes (Table 1). Because the two Hsp60 variants differed by 13 / 211 base pairs (substitutions), in addition to a 3 bp indel in one of the isoforms, we assume that these are different gene variants (denoted Hsp60a and b) and not different alleles. In all assays, the eukaryotic initiation factor 4A gene served as housekeeping gene, which has previously been tested for temperature sensitivity by Ransbotyn & Reusch (2006).

Table 1 Putative *Z. marina* genes associated with heat stress based on their putative Swiss-Prot homologue, and their homology to genes of *Arabidopsis thalina* (TAIR database) and *Oryza sativa* (TIGR database). For Genbank accession numbers of the underlying EST reads, and for molecular ecology resource numbers, refer to Table I-S1 (Appendix)

Gene name/abbreviation	function	Swiss-prot best hit	A.thaliana best hit	O. sativa best hit
Hsp81/hsp81	Molecular chaperone	sp | Q07078 | HSP83_ORYSJ	AT3G15353	LOC_Os05g11320
Hsp80/hsp80	Molecular chaperone	sp|P36181|HSP80_SOLLC	AT5G56030	LOC_Os09g30439
Hsp70/hsp70	Molecular chaperone	sp|P09189|HSP7C_PETHY	AT1G56410	LOC_Os11g47760
Hsp60 Isoform1/hsp60a	Molecular chaperone	sp|Q05046|CH62_CUCMA	AT2G33210	LOC_Os10g32550
Hsp60 Isoform2/hsp60b	Molecular chaperone	sp|Q05046|CH62_CUCMA	AT2G33210	LOC_Os10g32550
10 kDa chaperonin/10 kDa	Molecular chaperone	sp|O65282|CH10C_ARATH	AT5G20720	LOC_Os09g26730
Chaperon Protein DNA J1/DNAJ1	Molecular chaperone	sp|P30725|DNAJ_CLOAB	AT5G16650	LOC_Os02g46640
Universal stress protein/stress_prot	Scaffolding	No hit	AT1G10110	LOC_Os12g09089
70 kDa peptidyl-prolyl isomerase/PPIM1	Catalytic Activity	sp|Q43207|FKB70_WHEAT	AT4G10390	LOC_Os08g41390
Luminal binding protein/BIP	Luminal binding Protein	sp|Q03684|BIP4_TOBAC	AT5G42020	LOC_Os02g02410
Metallothionein protein type 3/MT3	Capture of trace elements	sp|Q40256|MT3_MUSAC	AT4G30160	LOC_Os05g51640
Copper chaperone/Cu_chap	Protein folding	No hit	AT3G56240	LOC_Os12g34850

The ratio of the amount of target gene mRNA to the amount of housekeeping gene mRNA was assessed with quantitative real-time PCR (qPCR) on a StepOne- Plus Cycler (Applied Biosystems, USA) using the Fast SYBR Green QPCR Master Mix (Applied Biosystems). qPCR reaction details are given in Table I-S1 (Appendix). Amplification efficiency of the 12 PCR assays was assessed through linear regressions of standard curves with 6 two-fold serial dilution points starting with a dilution of 1:10. Efficiency was calculated from the slopes of the threshold cycle (Ct) vs. concentration [cDNA] with the equation (I):

$$E = 10^{-1/\text{slope}} \qquad \text{(I)}$$

PCR efficiencies were always >1.90, all R^2 were > 0.95. All samples were duplicated on different plates. Technical variation (= varT) was calculated as the mean of the variation in housekeeping gene expression (varT = 0.15). If duplicates on the two different plates varied more than 0.45 (= 0.3 + varT) an additional triplicate was measured, which allowed outlier identification. Relative treatment gene expression values were calculated as:

$$-\Delta CT = CT\ (housekeeping\ gene) - CT(target\ gene) \quad (II)$$

$$-\Delta\Delta CT = -\Delta CT(treatment) - (-\Delta CT(control)) \quad (III)$$

Note that a negative treatment gene expression is possible and translates to a lower gene expression in the treatment compared to the control. A RNA pooling approach identified two genes that never revealed altered gene expression in any of the three populations, a metallothionein gene and a copper chaperone which were therefore excluded from replicated Q-PCR assays (Fig. I-S4, Appendix I-S4).

Data analysis

Assumptions for normality of all response variables were graphically examined. Tests were conducted accordingly using the software 'R' (R Development Core Team 2009) or JMP 6 (SAS Institute Inc., USA). Growth rates and shoot count were analysed with a general linear model constructed based on Underwood (1990, p 358 ff) with the fixed factors 'population', 'temperature' and 'time point' and the random factor 'tank' nested into the temperature treatment. When appropriate, Bonferroni-corrections for multiple testing were applied.

Multivariate statistics were used to infer differences in the entire expression pattern of 10 heat stress associated genes identified in a pooling approach (see Appendix I-S4). We calculated relative expression values following the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) for reasons of model simplification. To evaluate the joint effects of population affiliation and time point, a multivariate analysis of variance (MANOVA) on $-\Delta\Delta CT$-values was performed.

Relative Expression data ($2^{-\Delta\Delta CT}$) were fourth root transformed based on recommendations by Clarke et al. (2006) to allow the comparison of very low and high values on the same scale. An analysis of similarity (ANOSIM) based on the Bray-Curtis

matrix was then performed with the software PRIMER v6 (Clarke & Gorley 2006). This method assesses differences in expression patterns for the different populations at the different time points. A similarity analyses (SIMPER, Clarke & Gorley 2006) was then performed to identify genes contributing most to differences between treatment groups (see Jäger et al. 2007; Eizaguirre et al. 2009).

To assess differences in the variation of the heat stress response, σ^2-values (i.e. the variance) of relative expression data were calculated for each population and each of the 10 responsive genes. As σ^2-values were not normally distributed, we performed a Kruskal– Wallis test with the factor population.

Results

Population differentiation and diversity

Population differentiation measured as Wright's fixation index F_{ST} at neutral genetic markers between both Danish populations was substantial (F_{ST} = 0.08; p = 0.0001), but, as expected, considerably smaller than genetic differentiation between the southern and northern populations. (F_{ST} = 0.27 and 0.18 for Ebeltoft and Doverodde respectively, all p < 0.0001). Averaged over the 4 microsatellite markers employed, the population-wise heterozygosites were high (0.59 in Ebeltoft, 0.73 in Doverodde, both Denmark; 0.77 in Gabicce Mare, Italy). In total, 37, 18 and 58 different genotypes could be identified at the 10 sampling spots of 50 cm in diameter within the meadows for Ebeltoft, Doverodde (northern) and Gabicce Mare (southern population) respectively. In terms of mean number of clones detected per spot (50 cm diameter) this corresponds to 4.4 ± 0.4 standard error (SE), 2.8 ± 0.6 and 6.4 ± 0.7 SE for Ebeltoft, Doverodde and Gabicce Mare, respectively. No genotype was found twice or more at any of the sampling spots. During planting we combined samples coming from 8 to 10 spots at each donor location. As a result, the desired clonal diversity in the experiment was maximal for all populations and experimental boxes (i.e. two per tank per population).

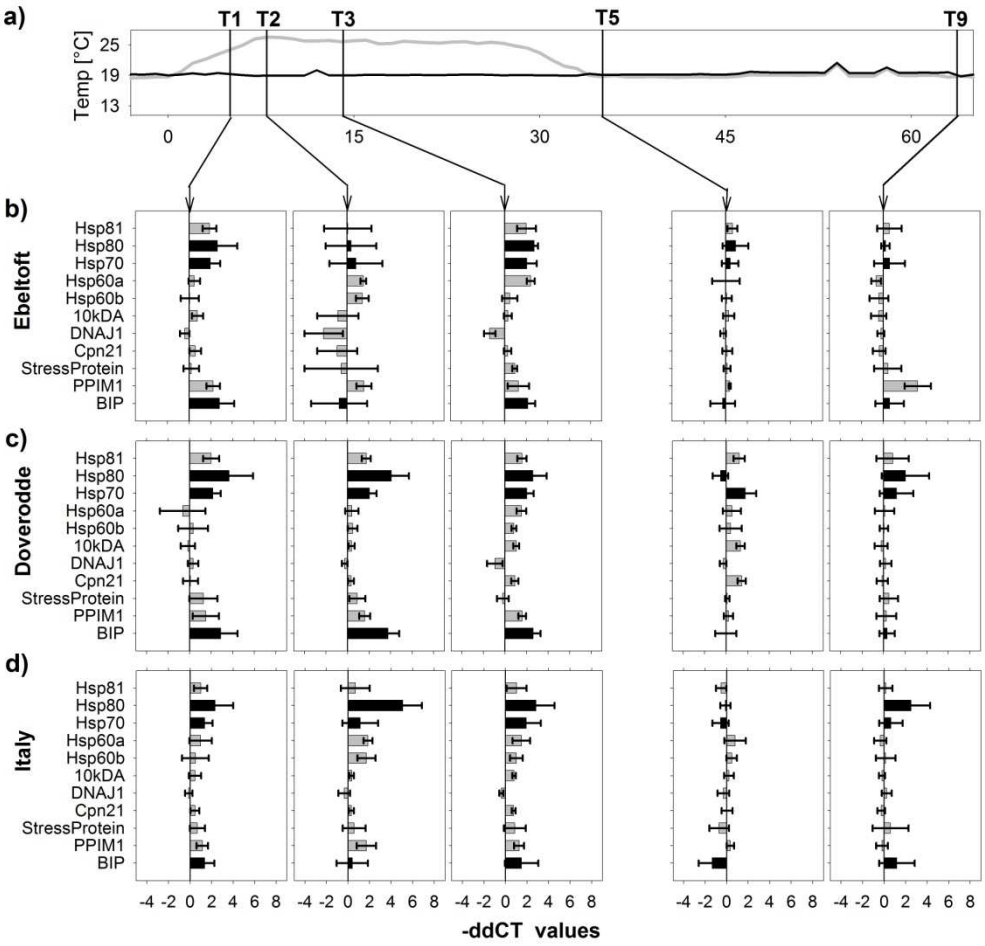

Fig. 2 Relative gene expression in the seagrass *Z. marina*; -ΔΔCT values of 10 stress associated genes that were induced relatively tothe control treatment. For comparison, the temperature course of the experiment is given in panel (a) (grey: heat treatment, black: control) and RNA sampling time points T1–T9. Panels (b)–(d) depict mean -ΔΔCT values ± 1 SE (n = 5) for Ebeltoft, Doverodde (northern population) and Italy (southern population), respectively. Gene expression was quantified at three time points during the simulated heat wave (T1–T3), directly after the heat wave (T5) and after 4 weeks of recovery (T9). Gene abbreviations are indicated on the y-axis (see Table 1 for full names); the three genes explaining most of the treatment differences (Hsp80, Hsp70 and BIP) are highlighted in black.

Phenotypic variables

Leaf growth rates were not influenced by temperature but showed significant differences among populations and time points as well as for their interaction (Table I-S2, Appendix). Mean growth rates ranged around 0.98 cm⁄ 24 h (± 0.05 cm SE) throughout the experiment (data not shown), which is approximately equal to field measures (see I-S7, Appendix) but lower than in another short-term experiment in the AQUATRON (I-S3, Appendix).

Relative to the control, treatment populations lost between 12% (Doverodde) and 35% (Ebeltoft) of their shoots throughout the heat wave, a decrease that was not different among populations as indicated by a nonsignificant interaction term 'population · temperature' (Fig. 1 and Table I-S2, Appendix). Even the southern population (Gabicce Mare), which overall performed best in the AQUATRON experienced a shoot loss of 25% in the warm treatment (Fig. 2). Interestingly, shoot reduction came with a delay in all populations. Directly after the heat wave (T5), shoot counts started to decrease in the heat stressed treatments, a difference that became statistically significant only during recovery at time points T8 and T10 (ANOVA; $F_{1,71} = 14.57$, $p = 0.0034$; $F_{1,71} = 14.47$, $p = 0.0035$ for T8 and T10 respectively).

Gene expression analysis

Qualitatively, many of the stress associated genes revealed positive $-\Delta\Delta$CT-values and hence, upregulation during the heat wave (Fig. 2). Among the genes with the highest fold-induction were one Hsp80 gene and a luminal binding protein (Bip), with a fold-induction during the heat wave of up to 13-fold (Bip) and 34- fold (Hsp80). Because we had no a priori hypothesis as to which of the target genes are most important to describe the heat stress response of Z. marina, our analyses started with multivariate tests, which also minimized the chances to commit type 1 errors. Gene expression relative to the control treatment (i.e. $-\Delta\Delta$CT) differed markedly among populations and time points (MANOVA, all $p < 0.0001$). More importantly, the 'temperature · population' interaction was also highly significant (all $p < 0.0001$), indicating that expression profiles are highly time point- and population-specific (see S5, Table I-S3, Appendix).

In one of the northern populations (Ebeltoft), we identified considerable variance among the individuals in terms of gene expression at the beginning of the heat wave (T2, Fig. 2). Accordingly, σ^2-values of the relative treatment gene expression ($-\Delta\Delta$CT) among the five Ebeltoft genotypes were significantly higher compared to both other populations (Kruskal–Wallis test, $p = 0.017$).

Time course of gene expression

The RNA pooling approach revealed that gene expression at the end of the heat wave (time point T4) was highly correlated with gene expression in the middle of the heat wave (T3) ($p < 0.0001$, $R^2 = 0.97$, Fig. I-S6, Appendix). This provided a rationale to only include T3 in the more extensive qPCR-assessment with 5 replicated genotypes for each 'temperature · population' combination.

In order to assess differences in the expression profile during different time points of our experiment, relative treatment gene expression values were averaged over populations for single time points. Significant differences between the theoretical control (T0; $-\Delta\Delta CT = 0$), initial heat stress expression profile (T1 & T2), heat stress expression profile (T3), recovery profile (T5) and long-term damage profile (T9) were detected using pair wise tests in an ANOSIM analysis (Table I-S4, Appendix and Fig. 3). We found a marked and statistically significant up-regulation in expression of heat stress related genes in plants immediately after exposure to the heat wave. At the end of the heat wave (T5), gene expression recovered quickly to reach control values apparent as $-\Delta\Delta CT$-values not very different from zero (Fig. 2). Interestingly, after 4 weeks of recovery, only those plants previously exposed to a heat wave showed again a significant up-regulation in heat stress related genes compared to control plants (Fig. 3 and 4).

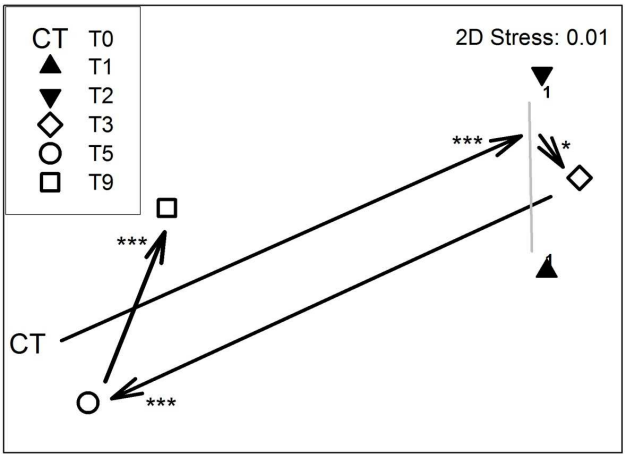

Fig. 3 Gene expression in the seagrass *Z. marina* during and after a heat wave. Multi-dimensional scaling (MDS) after ANOSIM analyses based on mean $-\Delta\Delta CT$-values averaged over populations. Each time point is depicted by a x–y-coordinate, with the distance to each other proportional to the similarity of the expression profile. Arrow labels depict the significance level (***highly significant difference between points, $p \leq 0.001$), *significant difference between points, $p \leq 0.05$). Points sharing the same number (1) are not statistically different (grey line) (for statistical analysis see Table I-S4, Appendix).

Population specificity of expression profiles

An analysis of single time points, now considering populations as factor, revealed population-specificity of expression profiles (ANOSIM analyses on -ΔΔCT values) at different time points. All three populations responded differently to the heat wave already early in the course of the experiment (T1: ns; T2: p = 0.002) and continued to display significantly different expression profiles throughout the heat wave (T3: p = 0.003). After temperatures returned to control conditions (T5), expression profiles still were significantly different (p < 0.004), with a trend lasting 4 weeks into the recovery period (p = 0.041) (Table 2 and Fig. 4).

Table 2 Pair-wise comparison of relative gene expression (-ΔΔCT) in the seagrass *Z. marina* among populations at five time points during and after an experimental heat wave using ANOSIM. Global R and significance values for single time points (T1–T9) and significance values for pair wise population comparisons at each time point are given

	Global *R*	**Global *P***	**Pair wise tests**	**DV**	**IT**
T1	0.06	0.24	EB	0.48	0.24
			DV		0.16
T2	0.32	**0.002**	EB	**0.008**	0.18
			DV		**0.016**
T3	0.26	**0.003**	EB	**0.016**	**0.008**
			DV		0.19
T5	0.31	**0.004**	EB	**0.032**	0.10
			DV		**0.008**
T9	0.16	0.041	EB	0.11	**0.008**
			DV		0.79

Significant differences are highlighted in bold; EB, Ebeltoft;DV, Doverodde; IT, Italy.

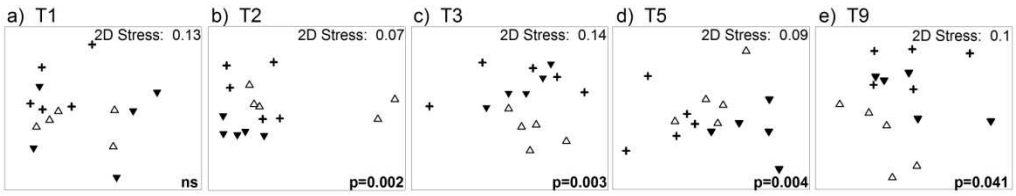

Fig. 4 Population-specificity of heat stress gene expression over time in the seagrass *Z. marina*; MDS based on relative gene expression (-ΔΔCT) for time points T1 (a) to T9 (e). For the position of time points see Figs 1 and 2. The significance values of the underlying ANOSIM analysis are given, testing the null hypothesis of no gene expression difference among populations at single timepoints. Each symbol represents the gene expression vector of 10 genes as an x–y-coordinate of one single genotype, with the distance to each other proportional to the similarity of the expression profile. Δ, Ebeltoft; ▼, Doverodde (northern populations) and +, Italy (southern population).

Contribution of genes to the treatment effects

In all three populations, only a small number of genes contributed to approximately half of the differences in total multivariate gene expression between populations and time points. The most consistent candidates across the three populations were Hsp80, Hsp70 and Bip which together contributed ~50% to the differences between treatments in a SIMPER analysis (Table 3). Averaged over all time points, the mean pair-wise dissimilarities per gene among all three possible population comparisons was of similar magnitude, ranging from 8.26 to 9.28%. The same magnitudes of dissimilarity were obtained when all five time points are considered separately (data not shown).

Table 3 Contribution of single heat stress genes to total gene expression differences in the seagrass *Z. marina* between populations (assessed as -ΔΔCT-values), given for all three populations after SIMPER analysis

	Ebeltoft-Doverodde	**Ebeltoft-Italy**	**Doverodde-Italy**
Average dissimilarity	**9.23**	**9.26**	**8.62**
Hsp80	**20.43**	**23.42**	**20.59**
BIP	**15.28**	**12.5**	**17.38**
hsp70	**11.23**	10.66	**12.82**
PPIM1	10.51	**11.36**	5.75
StressProt	8.84	9.63	8.88
Hsp81.3	8.56	9.55	10.12
Hsp60a	7.52	6.73	8.92
Hsp60b	6.15	6.30	7.16

Genes contributing most to the multivariate difference are displayed in bold. For full gene names, see Table 1.

Discussion

In the marine realm, it is largely unknown how ecologically important foundation species respond to global warming, possibly with the exception of reef-building corals (e.g. Rodriguez-Lanetty et al. 2009; Voolstra et al. 2009; Desalvo et al. 2010). Using a controlled common stress garden experiment, we assessed differences in thermal tolerance to a 3-week heat wave, including a recovery phase in three populations of the seagrass *Z. marina*, two from northern and one from southern European shores.

Our approach does not permit to explicitly test for local adaptation, as this would require exposing replicated pairs of southern and northern populations to a heat wave (Kawecki & Ebert 2004). However, we provide first important insights into the population specificity of the expression of stress-associated genes. To our surprise, each population showed a specific gene expression profile during and after the heat stress period, supported by several different multivariate analyses. Even the northern populations that are exposed to a largely similar thermal regime were more different to each other than we expected. Because we acclimatized plants over four weeks and exposed them to identical environmental conditions, our results are consistent with the idea that gene expression is at least partly heritable. Although we cannot exclude maternal and other carry over effects by the original environment, we suggest that the observed population specificity in gene expression is the result of locally varying selection regimes.

In contrast to gene expression, all three populations suffered equally from shoot loss during the course of the experiment which highlights the importance of our findings for the population persistence of European *Z. marina* in the face of global change even in southern populations. On the other hand, after the first recording in 1999 (Nastro 2004) the Gabicce Mare population persisted in recent years although water temperatures in the four preceding years regularly exceeded 26 °C during summer (Fig. I-S1C, Appendix).

Although our study is the first in marine organisms that included a stress recovery period of more than 30 days, our experimental duration is short compared to the entire life cycle of a seagrass, let alone the lifetime of a clonal plant. We thus suggest that in our experiment, genetically based local adaptation with respect to thermal regime was undetectable because it would have required a much longer

experimental duration to encompass important demographic processes, such as flowering, seed set and seedling survival.

With respect to gene expression, our core hypothesis was that differentially regulated stress genes among populations may indicate thermal adaptation (reviewed in Gibson 2008; Michalak et al. 2001). Our data tell a more complex story. While we found similar responses at the phenotypic level in terms of shoot losses and growth rates, the underlying gene expression pattern differed among all populations over time (Table 2). We thus find that an identical phenotype in terms of leaf growth and shoot loss is associated with different stress gene expression patterns. Interestingly, at the beginning of the heat wave, genotypes in one of the northern populations (Ebeltoft) revealed a markedly higher individual variance in gene expression compared to both other populations, possibly indicating genetic variation for gene expression within populations. It will be interesting to conduct similar experiments replicating on the genotypic level to assess within-population differentiation among genotypes and hence, the adaptive potential of local populations (Oleksiak et al. 2002). After four weeks of recovery, carry over effects in the gene expression profile re-appeared, resembling the initial stress response at the beginning of the heat wave. This molecular finding is consistent with a pronounced delay in shoot loss observed here and in previous field studies (Reusch et al. 2005) and experiments (Ehlers et al. 2008). Why the heat stress damage comes with such a delay is unknown and may relate to a deficit in assimilate storage that only becomes apparent later, in line with findings of an impaired photosynthetic performance of the plants under heat stress (G. Winters et al. unpublished data).

We found that 10 out of 12 putatively stress associated genes were induced relative to the control treatment during at least one time point during the heat wave in a marine plant. This is remarkable, as the putative functional role of all candidate genes for the heat stress response was inferred based on gene ontology categories solely developed in experiments with terrestrial plants. Our arguably small subsample of genes thus suggests an extensive overlap in the genetic repertoire to cope with temperature stress in angiosperms growing in two very different environments. Another expectation was that in an aquatic habitat, the time course of induction of heat shock genes such as Hsps should differ from terrestrial counterparts, as heat waves come more slowly, but persist longer than on land. Indeed, in *Z. marina* during a week-long heat stress, several genes remained up-regulated, whereas the

associated homologues in *A. thaliana* are no longer upregulated after 24–48 h (I-S6, Table I-S5, Appendix).

Among the genes that contributed most to the treatment effects were two classical Hsps (Hsp70 and Hsp80) that are known to play an important role in the stress response in many organisms (reviewed in Sorensen et al. 2003). Importantly, in our experiment, not all Hsp-congeners were equally important for the overall heat stress gene response. For example, one Hsp81 gene and 2 Hsp60-gene isoforms contributed relatively little to the multivariate discrimination among time points and populations (Table 3). Average fold-differences among the topmost discriminatory genes varied considerably, with maximal fold-changes found in Hsp80 (≤ 34-fold at the beginning of the heat wave T2), followed by Bip and Hsp70 (13 and 4.4-fold, respectively). Thus, if we were to design an essay as an early warning stress indicator (Hoffmann & Daborn 2007), we would use those 2 candidates, Bip and Hsp80, that combine discriminatory power (SIMPER analysis) and high sensitivity, as revealed by high fold-changes. Currently, we lack the physiological knowledge pertaining to why some of the responsive genes are contributing more to the heat stress response than others. Interestingly, we found that a luminal Bip is important to explain different expression profiles in Z. marina. Bip is known to influence leaf senescence under drought stress in soy and tobacco (Valente et al. 2009). Among our target genes were two different representatives (10 kDa and DNAJ1) of small Hsps, the most prevalent genes responsive to thermal stress in terrestrial plants (e.g. Wang et al. 2004). Contrary to our expectations, these genes contributed less in explaining differences in the expression profiles of *Z. marina*. Taken together, these findings suggest functional divergence in these genes driven by novel selection pressures for submerged plants that are living without drought or rapid temperature changes.

The application of gene expression measurements in order to study the physiological basis of thermal tolerance is an expanding field in marine Molecular Ecology (e.g. Heise et al. 2006; Whitehead & Crawford 2006a; Kuo & Sanford 2009; Morley et al. 2009; Pearson et al. 2009; Lago-Leston et al. 2010). In order to make inferences from measurements of mRNA abundance via qPCR to genetically based differences in gene regulation, measurements must be taken in a controlled environment to account for environmental variation (Whitehead & Crawford 2006a; Kuo & Sanford 2009; Lago-Leston et al. 2010). Moreover, as genotype by environment interactions are the essence of local adaptation, an additional requirement is to

perform tests under the appropriate stressor compared to a control (Kawecki & Ebert 2004; Gibson 2008; Hodgins-Davis & Townsend 2009). This can only be done in a common stress garden, rather than a simple common environment. Unfortunately, with our experimental design we cannot rule out that some of the gene expression profiles are only the result of long-term carry over effects. This can only be circumvented if organisms from different origin are kept in the laboratory for multiple generations. As one exception, Kuo & Sanford (2009) acclimated snails (*Nucella canaliculata*) from different populations over two generations in the lab before exposing them to a thermal gradient, and found that differences in upper thermal limits likely had a genetic basis. While desirable, such an approach is not possible in a long-lived clonal plant such as *Z. marina*.

To summarize, our study highlights the importance of realistic stress and recovery scenarios when studying the response of marine organisms to global change scenarios. As such, our results support several recent claims that recommend an enhanced use of molecular approaches in marine ecology (Johnson & Browman 2007; Hofmann & Gaines 2008), especially to uncover the genetic basis of more traits that are fitness-related under global change (Reusch & Wood 2007). This applies for seagrasses in particular (Procaccini et al. 2007), as to date, the genome of Z. marina is being sequenced by the DOE Joint Genome Institute (http://www.jgi.doe.gov/). This will expand the molecular tool box for this important marine ecosystem engineering species, thus facilitating answers to open ecological questions by a careful integration of 'new' molecular methods and 'old' classical ecological approaches.

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Chapter II

The edge of a tight eelgrass meadow in the Baltic

Chapter II

Transcriptomic resilience to global warming in the seagrass *Zostera marina*, a marine foundation species

Abstract

Large-scale transcription profiling via direct cDNA sequencing provides important insights as to how foundation species cope with increasing climatic extremes predicted under global warming. Species distributed along a thermal cline, such as the ecologically important seagrass *Zostera marina*, provide an opportunity to assess temperature effects on gene expression as a function of their long-term adaptation to heat stress. We exposed a southern and northern European population of Zostera marina from contrasting thermal environments to a realistic heat wave in a common-stress garden. In a fully crossed experiment, eight cDNA libraries, each comprising ~125 000 reads, were obtained during and after a simulated heat wave, along with nonstressed control treatments. Although gene-expression patterns during stress were similar in both populations and were dominated by classical heat-shock proteins, transcription profiles diverged after the heat wave. Gene expression patterns in southern genotypes returned to control values immediately, but genotypes from the northern site failed to recover and revealed the induction of genes involved in protein degradation, indicating failed metabolic compensation to high sea-surface temperature. We conclude that the return of gene-expression patterns during recovery provides critical information on thermal adaptation in aquatic habitats under climatic stress. As a unifying concept for ecological genomics, we propose transcriptomic resilience, analogous to ecological resilience, as an important measure to predict the tolerance of individuals and hence the fate of local populations in the face of global warming.

Introduction

Global climate change is imposing increasing stress on many organisms. Thus, one central question in ecology and evolution is how species cope with such environmental challenges (Etterson & Shaw 2001, Root et al. 2003, Hoffmann & Sgrò 2011, Lubchenko 1998). Global climate change is characterized by both the change in mean variables and the increase in extreme events such as heat waves, droughts, and heavy precipitation (Easterling et al. 2000). These extreme events, in particular, strongly impact ecosystems and associated species (Easterling et al. 2000, Walther et al. 2002). In habitat foundation species (sensu Dayton 1972), such as corals, trees, and seagrasses, entire ecosystems depend on the stability and performance of populations of single species (Jones et al. 1994). Therefore, their persistence in the light of climate change is of particular interest. Among these habitat foundation species are the 60 or so species of seagrasses (Den Hartog 1974), monocotyledonous plants which form the basis of productive marine ecosystems, providing habitat and nursery grounds for associated species (Hughes et al. 2009b). Seagrass- based ecosystems alter the physical environment by stabilizing the sediment, nutrient fixation, and current modification (Larkum et al. 2006), sometimes exceeding the ecosystem services provided by coral reefs (Costanza et al. 1997). Accelerating global declines of seagrasses therefore are of major concern (Waycott et al. 2009). Currently, it is unclear how much ocean warming contributes to seagrass decline, potentially exacerbating the other known anthropogenic factors that negatively impact seagrass beds such as eutrophication, fishing, mechanical destruction, and aquaculture (Waycott et al. 2009).

We focus here on the effects of extreme summer sea-surface temperature on eelgrass (Zostera marina), a widespread and often locally dominant seagrass species of the northern hemisphere (Den Hartog 1974). Field surveys and experimental studies suggest that high water temperatures (≥25 °C) during summer heat waves increasingly threaten seagrass performance and survival in northern European seagrass beds (Nejrup & Pedersen 2008, Ehlers et al. 2008, Greve et al. 2003, Reusch et al. 2005). In contrast, genetically divergent Z. marina populations (Olsen et al. 2004, Reusch et al. 2000) persist in Mediterranean bays and lagoons where summer temperatures frequently exceed 26 °C (Bergmann et al. 2010), suggesting enhanced thermal tolerance of the high summer temperatures in these locations. Together

these data emphasize the importance identifying the physiological and genetic basis of thermal tolerances across species' distributions (Etterson & Shaw 2001, Davis & Shaw 2001) and addressing whether limits to distributions can evolve or represent fundamental constraints (Kellermann et al. 2009).

Global gene-expression profiling is one emerging approach toward understanding these differences in tolerance, because thermal tolerance often is reflected in the differential expression of particular genes under thermal exposure, and such variation between populations often is indicative of local adaptation (Hoffmann et al. 2003, Whitehead & Crawford 2006a, Whitehead & Crawford 2006b, King & Wilson 1975, Ferea et al. 1999, Sorensen et al. 2007). Studying populations along a thermal gradient, for example the northern and southern European seagrass populations, is particularly instructive, because we can test the basic hypothesis that differential expression among localities under heat-stress conditions reflects thermal adaptation (Reusch & Wood 2007). Few studies have applied large-scale gene expression in natural populations along an environmental gradient (but see Whitehead & Crawford 2006a, Oleksiak et al. 2005, Stillman & Tagmount 2009, Polato et al. 2010), and we are unaware of studies using next-generation RNA sequencing technologies that do not require a priori genomic/transcriptomic information for digital gene-expression analysis (Torres et al. 2008, Bräutigam & Gowik 2010). Still fewer studies have covered the recovery phase after an extreme event explicitly, and those that have done so have considered only a short time scale of a few hours to days (e.g. Swindell et al. 2007, Frank et al. 2009, Chauhan et al. 2011). We also are not aware of any study on ecologically important foundation species, which often are not genetic/genomic model species.

To bridge this gap, we used 454 direct cDNA sequencing (Bräutigam & Gowik 2010, Tautz et al. 2010) for a global assessment of the transcription profiles of heatstressed and nonstressed eelgrass (*Z. marina*) plants from two contrasting locations. The experimental heat stress mimicked an actual heat wave that struck Europe in 2003 (Fig. II-S1, Appendix) (Schär et al. 2004) and led to considerable mortality in northern European seagrass populations (Ehlers et al. 2008, Reusch et al. 2005). Our goal was to identify putative genes and molecular functions involved in adaptation to the specific local conditions (Whitehead & Crawford 2006a, Cheviron et al. 2008), with special attention to a joint analysis of both the acute response to heat stress and the recovery phase. Although we expected classical heat-shock proteins

(Hsps) and chaperones to play an essential role during the acute phase of the heat wave (Feder & Hofmann 1999, Wahid et al. 2007, Kotak et al. 2007), longer-term effects on the cellular metabolism, including increasing expression of proteins involved in protein degradation needed for the turnover of irreversibly damaged proteins, may be as critical as changes during the stress event (Aro et al. 1993, Giardi er al. 1996). Such assessments are possible only with a more comprehensive gene-expression analysis that goes beyond available target-gene approaches (Bergmann et al. 2010).

Material and methods

Study Species and Experimental Setup

Eelgrass (*Z. marina*) plants were collected from two different populations located in Doverodde, Denmark (Limfjord, 56°43.07'N 8°28.45'E, hereafter "north") and Gabicce Mare, Italy (Adriatic Sea, 43°57.97'N 12° 45.86'E, hereafter "south") in early spring 2008 and transported within 48 h to the laboratory. At each location ~30 leaf shoots were sampled from each of ~15 sampling spots to collect several different genotypes that were identified via microsatellite genotyping (Bergmann et al. 2010). Plants were planted in a mesocosm facility at the University of Münster (Münster, Germany) that has been described in detail elsewhere (Bergmann et al. 2010). In brief, the experimental set-up consisted of two temperature-controlled flumes, each with six large 1-m^3 tanks filled with artificial seawater and silicate sediments to a height of 10 cm. Plants were grown at 31 practical salinity units and under light-saturated conditions (~400 µmol photons s^{-1} m^{-2}). Plants were allowed to acclimatize for ~30 d while the temperature was raised slowly from 14 °C (collecting temperature) to 19 °C, the control temperature during the heat-wave treatment (Fig. II-S1, Appendix).

Heat-Wave Simulation

The heat-wave simulation followed a common-stress garden design. Half of the experimental units were kept at a water temperature of 19 °C after acclimation, and the other half was subjected to a simulated heat wave, where water temperature

was increased by 1.5 °C/d until it reached 26 °C. This temperature was maintained for 3 weeks, followed by a temperature decrease of 1.5 °C/d until the water temperature reached 19 °C again (Fig. II-S1, Appendix). The temperature profile closely followed the summer heat wave of 2003 (Reusch et al. 2005).

RNA Preparation, 454 Library Construction, and Sequencing

RNA samples were taken from eight conditions: the northern vs. southern population, under heat stress vs. control conditions, with samples taken at two time points during the experiment, in the middle of the heat wave, and 1 d after return to control values (Fig. II-S1, Appendix). For RNA extraction an ~2-cm-long section from a young, growing leaf was cut from a randomly chosen plant from each condition and replicate, cleaned, frozen by dipping into liquid nitrogen, and immediately ground in a ball mill, followed by RNA extraction with the Invisorb RNA plant HTS 96 extraction kit (Invitek). For each condition RNA samples were pooled from six to eight genotypes. cDNA libraries were constructed using the Clontech SMART cDNA synthesis kit (Invitrogen). Firststrand synthesis was performed with ~0.5 µg total RNA for first-strand synthesis via anchored oligo(dT) priming followed by 15 amplification cycles. Sequencing libraries were constructed based on 3 µg of obtained cDNA. Every library was sequenced on a quarter of a slide using physical library separation with the 454 Genome Sequencer FLX using the Titanium chemistry (Roche and 454 Life Sciences).

EST Preprocessing and de Novo Transcriptome Assembly

After removal of the adaptor sequence, EST reads were quality trimmed with standard settings of the 454 Genome Sequencer FLX proprietary software (Roche) software. These raw sequence data for the eight generated libraries were deposited at the Sequence Read Archive (accession no. SRP007220). cDNA primer contaminations introduced by the SMART cDNA synthesis kit (Invitrogen) were identified by CROSSMATCH (http://www.phrap.org/) using the parameters minmatch 10 and minscore 15.

Three different subsets of 454 Genome Sequencer ESTs originating from different *Z. marina* populations were assembled independently into contigs to minimize polymorphisms derived from multiple divergent genotypes for de novo

assembly. Subsequently they were combined in a final mapping assembly of the contigs of the northern and the southern population assemblies against the assembled contigs with the sequence reads obtained from a single *Z. marina* clone (Falkenstein, Germany) used as a backbone. De novo assembly was performed using MIRA (version 3.0.3, http://sourceforge. net/projects/mira-assembler/files/) using standard parameter settings in the accurate assembly mode (Chevreux et al. 2004). Note that the ESTs for the third assembly came from a single clone (Falkenstein, Germany, Baltic Sea, 54°24'N 10°12'E), comprised of 866838 Roche 454 Titanium EST reads (NCBI Sequence Read Archive, accession no. SRA002573) (Table II-S1, Appendix). The final assembly was created by mapping the contigs obtained from the two population assemblies against those from the single-clone assembly. For the second-stage assembly, MIRA was used in the mapping and accurate mode, also allowing the creation of new contigs.

Quantification and Verification of Library-Specific Gene-Expression Profiles

Differential gene expression for each of the eight experimental conditions was assessed by mapping cleaned sequence reads first to the final transcriptome assembly with BLASTN (Altschul et al. 1990) and then identifying tentative unigenes via annotation using the *A. thaliana* proteome, using TAIR9 (Swarbreck et al. 2008). We also mapped against rice *O. sativa* (Michigan State University Rice Annotation Project Release 6.0) using BLASTX. Our approach was verified via quantitative real-time PCR for a set of 18 genes. Log2 fold-changes between read counts of heat and control treatment and ΔΔCT values (Bergmann et al. 2010) for both populations in the middle of the heat wave were compared (Fig. II-S2, Appendix).

Identification of TDE Genes

Genes TDE in response to the heat treatment were identified via bootstrapping for all four pairwise comparisons of control vs. heat-stress treatment. A null model was created under the hypothesis that gene expression is not different in heat-stress and control treatment. Sequence reads were resampled with replacement from the expressed sequence read pool of the control library. For each of the 10,000 bootstrap replicates, reads were sampled until the number of reads in the respective

heat-stress library was attained. For each gene, the read count of the heat-treated library then was compared with the read distribution of the created null model to calculate empirical P values. P values were calculated with subsequent one-sided tests of the area outside the null distribution when assuming higher as well as lower gene expression. To correct for multiple testing, the false discovery rate (FDR) of α = 0.01 was used (Benjamini & Hochberg 1995); thus we expect only 1% of genes to be false positives per pairwise comparison and roughly 4% to be false positives on a per gene basis. At low absolute expression levels of a given gene (~40 reads per library comparison), a log2 fold-change of ~2 can be detected (Fig. II-S3, Appendix). Most indicator genes (215/234) identified in the analysis described below, on which our data interpretation relies, have absolute transcript abundances of ≥10 reads in at least one of the libraries involved (Fig. II-S3, Appendix).

Multivariate Analysis of Gene-Expression Profiles

The profiles of all TDE genes were subjected to multivariate analysis to detect similarities and differences in the transcriptomic response to treatments. Expression profiles were normalized for differences in library sizes (Table II-S2, Appendix) (Anders & Huber 2010) and scaled across all eight libraries to a mean of zero and an SD of one. PCA was performed with the R package VEGAN [(http://cran.r-project.org/web/packages/vegan/index.html) version 1.17–6].

The resulting groups then were tested with ANOSIM implemented into VEGAN. Euclidian distances were used to calculate similarities between libraries, with P values calculated based on 10000 permutations (D'haeseleer 2005). The identification of genes that were indicative for the identified grouping of libraries followed an indicator value analysis (De Càceres et al. 2010), calculating a correlation index for each gene between the given grouping and the expression values across the libraries. The method is implemented in the R package indicspecies (version 1.5.1). Subsets of indicator genes were functionally annotated via MapMan (Usadel et al. 2005). Abundant functional groups within those subsets of genes were tested formally for overrepresentation against the entire set of functionally annotated indicator genes using one-sided Fisher's exact test.

Results

Assembly and Gene Annotation

Our global gene-expression analysis using leaf tissue covered ~one-third of all genes of a typical flowering plant. The number of unique genes was highest in the larger reference clone assembly with 11135 gene identifications. The population-level assemblies identified 8673/8579 unique genes for the northern and southern populations, respectively (Table II-S1). We identified tentative unigenes by mapping de novo-assembled contigs against reference proteomes from *Arabidopsis thaliana* and *Oryza sativa*. Annotation success was high, with 76% and 78% of the *Z. marina* contigs yielding results with e-values $<10^{-4}$ against the *A. thaliana* proteome for the northern and southern population, respectively (Table II-S1). Often, more than one of the obtained de novo-assembled *Z. marina* contigs mapped against the same *Arabidopsis*-annotated tentative unigene. The underlying causes were evaluated in a small case study mapping all contigs against a set of 14 nonredundant *Z. marina* coding sequences downloaded from the National Center for Biotechnology Information (NCBI). The observed contig redundancy was caused by a combination of polymorphisms in gene sequences obtained from multiple genotypes in the libraries, sequencing errors, and contigs that mapped to different, nonoverlapping sections of reference genes. Interestingly, the annotation success against the phylogenetically closer monocotyledonous plant species *O. sativa* (rice) was very similar (Table II-S1, Appendix). We thus continued with *A. thaliana* as reference because it is the better-annotated plant species. When very lowly expressed genes (read count across libraries of ≤2) were eliminated, 5908 genes remained, corresponding to 104753–139020 EST reads for each library (Table II-S2, Appendix).

Validation of 454 Transcriptome Sequencing via Quantitative Real-Time PCR

Expression profiles obtained by direct cDNA 454 sequencing were validated using a sample of candidate genes measured in both populations during the heat wave using an independent method. We quantified expression levels of 18 genes in replicated genotypes (n = 5) using quantitative realtime PCR [via the delta-delta cycle threshold (ΔΔCT) method (Livak & Schmittgen 2001)] and compared these levels with

data obtained via direct cDNA sequencing (via $\log_2$ fold-change) (Fig. II-S2, Appendix). The two methods corresponded well, with correlation coefficients r = 0.812 and 0.807 for *A. thaliana* and *O. sativa* as reference proteome, respectively (P < 0.001) (Fig. II-S2, Appendix).

Multivariate Characterization of Gene-Expression Profiles

We identified genes that were tentatively differentially expressed (TDE) in response to the heat treatment by assembling sequence reads into contigs and then mapping those contigs against *A. thaliana* orthologs. We refer here to TDE genes because we do not wish to draw any conclusions based on particular genes; it is well known that genes identified in global transcription profiling need independent verification, for example using quantitative realtime PCR as described above. TDE genes were determined separately in all four library pairs (northern/southern population * during /after heat wave). Of 5908 genes, 1872 revealed evidence for significant changes in expression at least once and were kept for further analyses (Table II-S3, Appendix). TDE genes with ~40 mean read counts across libraries revealed fold-changes of ~2, which we consider appropriate, although with higher transcript abundance, smaller fold-changes were detected as significant (Fig. II-S3, Appendix). TDE genes were not interpreted at face value but only based on functional groupings that emerged from subsequent multivariate analyses. To identify patterns of similarity across all eight treatments (north/south * heat stress/control * during/after heat wave), a principal component analysis (PCA) of scaled expression profiles explained 41.84% of the variance and revealed three distinct clusters (Fig. 1). The largest cluster comprised libraries obtained from all four control conditions, along with the previously heat-stressed library of the southern population after the heat wave. A second well-defined cluster included the heatstressed libraries of both populations during the heat wave. As a third cluster, genes in the northern population upon termination of the heat wave showed the most divergent expression of all libraries. The grouping of libraries was supported by analysis of similarity (ANOSIM) of the scaled expression profiles of TDE genes (r = 0.7968; P = 0.0057). We also performed a similar PCA on a smaller subset of TDE genes. When applying a more stringent filter of ≥10 reads in at least one of the libraries revealing significant expression change, 1422 of the initial 1872 TDE genes remained. When analyzed as

above, full support of the three identified clusters in the PCA could be found (ANOSIM, r = 0.7112, P = 0.0059).

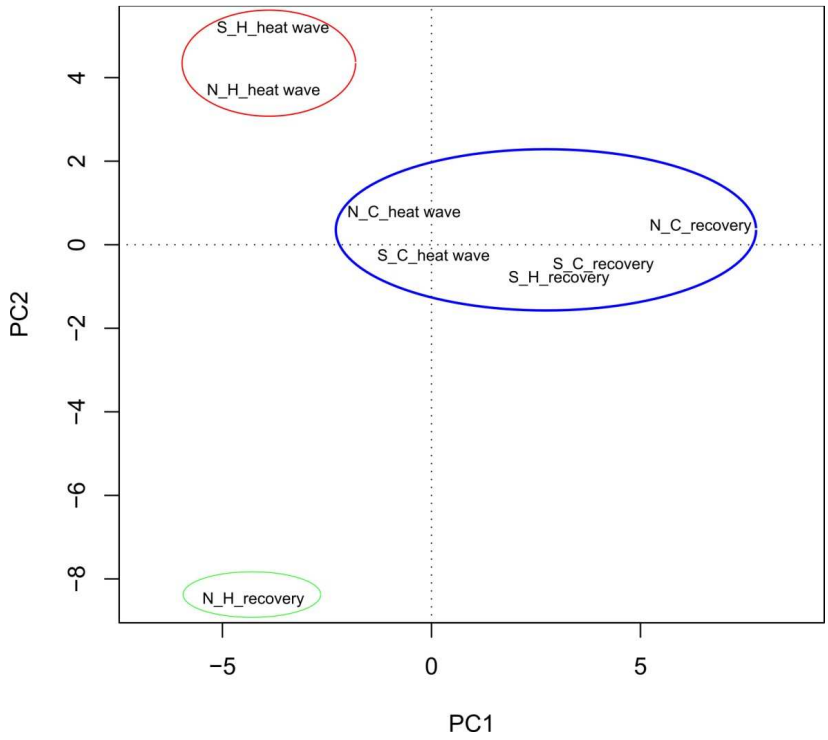

Fig. 1 Multivariate grouping of experimental libraries based on the expression profiles of 1,872 TDE genes using PCA. TDE genes between heat and control treatment were assessed for four library combinations (southern vs. northern population; within vs. after heat wave). PCA was performed on the scaled expression matrix of genes (mean = 0; SD = 1). Populations: northern (N), southern (S); heat treatment (H), control treatment (C); time points: acute heat and recovery. Groupings are indicated by color: blue, expression during control conditions; red, expression during heat stress; green, divergent expression during early recovery. A list of all TDE genes is found in Table II-S3, Appendix.

Identification and Functional Annotation of Indicator Genes

To identify TDE genes with the largest contribution to the identified grouping, an indicator gene analysis was conducted. Among the 234 TDE genes identified (all r ≥ 0.9 with above groupings; Table II-S4), 17 were specific for group 1 (the "control expression" group), with six indicating up-regulation and 11 indicating down-regulation of that group (Fig. 2). Group 2 ("during heat stress") was supported by the expression of 39 genes, 34 showing group-specific up-regulation and five showing up-regulation. Group 3 ("divergent northern recovery") contained 178 genes, 173 showing up-regulation and five showing down-regulation in comparison with the remaining groups.

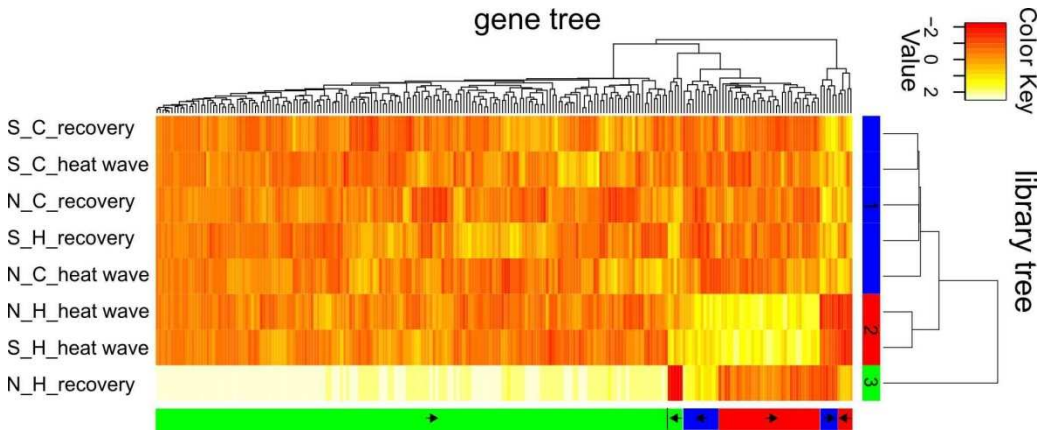

Fig. 2 Heatmap shows cross-correlation by treatment and similarity of gene-expression profiles of 234 heat-responsive genes that are strongly correlated (r ≥ 0.9) with the PCA-based clustering (Fig.1). x axis: columns display the cDNA libraries from the eight treatments, clustered by similarities among gene-expression profiles; y axis: each row displays the expression strength of a particular gene in the respective library, clustered by similarities across treatments. Expression strength was scaled for each gene across libraries (mean = 0; SD = 1). Values are color coded (white: highest expression strength; red: lowest expression strength). In combining library (treatment) and gene clustering, the following groups of genes are indicated: blue (1), control expression; red (2), during heat stress; green (3), divergent early recovery. Arrows along the gene order indicate up- and down-regulation of genes. Treatment codes are as in Fig. 1. Functional annotations of the six different gene sets that display characteristic up- or down-regulation of a group are shown in Fig. 3 and Fig. II-S4, Appendix; a detailed list is given in Table II-S4, Appendix.

The six different subsets of indicator genes (respective PCAbased group * up-/down-regulation) were functionally annotated via MapMan (47). The dominant functional group of up-regulated genes during heat stress was associated with the category “stress.abiotic.heat” (25% of all annotations) and consisted of various Hsps (Fig. 3A), which were significantly overrepresented (Fisher’s exact test; $P < 0.001$). Further categories were related to “protein” (22%) (belonging to the differing subcategories protein targeting, degradation, posttranslational modification, folding, and amino acid activation) and the categories “RNA. Regulation of transcription” (6%), “development” (6%), and “signaling” (6%). In contrast, Hsps played a only minor role during early recovery of the northern population (Fig. 3B). Here, genes of different putative functions were abundant. The category “protein.degradation” was most frequent (12%) and also was significantly overrepresented (Fisher’s exact test; $P = 0.034$). Other frequently identified genes were associated with categories “RNA.regulation of transcription” (9%), “protein.postranslational modification” (6%), and “signaling” (7%). The functional annotation of the remaining four groups of indicator genes can be found in Fig. II-S4, Appendix.

Fig. 3 Functional annotation of gene sets showing up-regulation in a specific group of libraries in comparison with the remaining groups. Putative functions of (A) up-regulated genes in group 2, during heat stress (5.6% of genes not annotated) and (B) up-regulated genes in group 3, divergent early recovery (24.1% of genes not annotated). Genes were annotated with MapMan categories and are presented via term clouds; the annotation frequency is proportional to word size. Gene categories that are significantly enriched (Fisher's exact test) are marked (*P ≤ 0.05; ***P ≤ 0.001).

Expression of Hsps During Heat Stress

We further scrutinized gene-expression patterns during the heat wave in both populations and searched for TDE genes that were annotated by MapMan category "stress.abiotic.heat" or directly with the key term "Hsp"; this search yielded 27 genes. The expression of these 27 genes during heat stress was highly correlated in the two

populations ($r = 0.97$; $P < 0.001$). Of these 27 genes, only two showed differential expression between populations during the heat treatment (Fig. II-S5, Appendix).

Discussion

In this study we examined a nearly complete transcriptomic response to global warming by a foundation species that structures an entire ecosystem, including a recovery phase after a realistic heat-wave scenario. The experimental genotypes came from two contrasting regions in the European thermal cline, the Adriatic Sea (southern population, Italy), and the Limfjord (northern population, Denmark).

After systematic data reduction, two salient findings of the transcription profiling were the similarity of gene expression during the heat wave and the strong divergence between the two populations shortly thereafter. During heat stress, in both the northern and southern population, the transcriptomic syndrome was dominated by the up-regulation of genes associated with the MapMan annotation "stress.abiotic.heat" (Fig. 3A), comprised mainly of classical Hsps. This finding is in line with studies suggesting that the expression of Hsps and molecular chaperones is correlated with thermotolerance and thermal adaptation (Feder & Hofmann 1999, Wahid et al. 2007, Kotak et al. 2007, Sorensen et al. 2003). Most previous studies, however, came from terrestrial organisms, and the duration of the experiment and the period of gene induction were, on average, 10-fold shorter (Swindell et al. 2007, Camejo et al. 2005, Tóth et al. 2005, Mittal et al. 2009, Ouyang et al. 2009, Hüve et al. 2011). Gene-expression studies in aquatic plants that experience more gradual temperature changes remain largely unexplored (but see Henkel & Hofmann 2008 and Pearson et al. 2009). Aquatic organisms experience smaller extremes in temperature that are dampened because of the high specific heat of the aqueous medium (Bergmann et al. 2010, Feder & Hofmann 1999). Once critical temperatures are attained, however, these conditions typically last longer in aquatic habitats, with no possibility for evaporative cooling. This gradual onset and prolonged duration of critical temperatures may explain, at least in part, why differences in expression between the two populations during the heat-stress treatment were modest, with only two of 27 Hsp genes revealing differences in expression between populations (Fig. II-S5, Appendix).

In contrast, both populations showed drastic differences in gene expression 1 d after termination of the heat wave. The expression profile of the southern population revealed considerable resilience and rapidly returned to control expression levels, but the transcription profile of the northern population diverged even further from all control treatments. The functional annotation of up-regulated genes in the northern population suggests a nonadaptive syndrome of failed metabolic compensation in the northern plants. Accordingly, up-regulated functions after the heat stress were dominated by “protein. degradation” and “RNA.regulation of transcription,” suggesting that proteins were damaged irreversibly and needed to be degraded and removed from the cell (Goldberg 2003). Similar up-regulation of protein degradation has been observed in other plant species under various stressors (Aro et al. 1993, Giardi et al. 1996, Degenkolbe et al. 2009). That the southern population returned so rapidly to control levels of gene expression demonstrates that temperatures >26 °C do not represent a fundamental limit to the distribution of *Z. marina* (Kellermann et al. 2009), a finding that is significant for seagrass conservation and ecology (Waycott et al. 2009).

The use of next-generation direct cDNA sequencing on the Roche 454 Titanium platform enabled us to perform global transcription profiling in a non-model foundation species (Bräutigam & Gowik 2010, Tautz et al. 2010), at the cost of having to pool RNA over individuals within treatments. The digital expression measurement on cDNA libraries of pooled genotypes was verified by quantitative real-time PCR. The quantitative real-time PCR measurements in 18 genes used biological replicates (n = 5) sampled in the same experiment (Fig. II-S2, Appendix). Because the level of concordance between the two methods was high ($r \sim 0.8$; $P < 0.001$), we conclude that the pooling of genotypes within treatment had only a small effect on the biological signal.

Upon de novo transcriptome assembly, our actual gene identification was guided by mapping to orthologs in reference proteomes in well-characterized plant species. Although de novo transcriptome assemblies have been performed on a variety of higher plants (e.g. Vega-Arreguín et al. 2009, Wang et al. 2009, Franssen et al. 2011a), currently used assembly software and assembly strategies are still in need of improvement, particularly when reference genomes are unavailable (Kumar & Blaxter 2010). In these cases, annotations against reference proteomes of related plant species, as was used here, are the second-best alternative. A reassuring result

was that gene identification success was largely congruent, whether we used amonocotyledonous (*O. sativa*) or dicotyledonous (*A. thaliana*) plant species as reference proteome; the former was phylogenetically closer than the latter to our target species (Anderson & Janssen 2009).

One key assumption of our common-stress garden approach is that the observed differences in gene expression in the two populations have a heritable basis, at least in part (Whitehead & Crawford 2006b, Sorensen et al. 2007, Stillman & Tagmount 2009). In allowing a relatively long acclimation period, we tried to minimize the likelihood that our common-stress garden design captures carry-over effects from the past environments of the experimental plants. However, environmental and epigenetic influences during the life history of the studied individuals cannot be ruled out completely (Wang et al. 2004, Rapp & Wendel 2005).

The transcriptional patterns observed here were in line with recent phenotypic measurements of photosynthetic performance using pulse amplitude-modulated fluorometry, in which southern *Z. marina* genotypes, in contrast to their northern counterparts, recovered to control values immediately after the heat wave (Winters et al. 2011). Because dramatic differences in gene responses were detectable only during recovery, we speculate that many previous studies investigating transcriptomic responses to acute stress missed critical gene-expression patterns. We posit that evolutionary ecology experiments addressing the physiological response among divergent populations should fulfill the following criteria: (i) inclusion of a reasonable acclimation phase; (ii) application of realistic stress scenarios including rate of increase, intensities, duration, and recovery; and (iii) inclusion of nonclassical stress genes to detect general deviation from normal cell homeostasis compared with a control treatment.

Because acute heat-stress responses were surprisingly similar among genotypes from two locations with widely diverging thermal conditions, we suggest that the transcriptomic patterns during recovery may be a better predictor as to how populations across latitudinal clines are adapted to thermal stress. As a unifying concept for ecological genomics, we propose transcriptomic resilience, describing the return to control levels of gene expression, analogous to ecological resilience, which describes the return of species abundance and performance to predisturbance conditions (Dayton 1972, Pimm 1991).

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Chapter III

Eelgrass in the Mediterranean Sea –

Seagrasses provide habitat for a variety of species

Chapter III

Genotypic variation in temperature stress response of eelgrass (*Zostera marina*) revealed in a common stress garden

Abstract

A prerequisite for evolutionary adaptation to and thus population persistence in the face of global warming is intra-specific variation in key traits important under the new selection pressures. Genetic variation among individuals that confers fitness advantages in the context of global climate change is currently understudied. We assessed variation in several physiological parameters as well as differences in gene expression of three previously identified indicator genes for heat stress (Bip, Hsp70 and Hsp80) between individuals of the marine engineering species Zostera marina. Replicates of five *Z. marina* genotypes of a Baltic population were exposed to a simulated three week summer heat wave of 26°C with a subsequent three week recovery phase at 19°C in a common stress garden. We followed optimum quantum yield, shoot count and leaf growth as well as RNA expression over the course of the experiment. We found that different genotypes have varying growth rates in control and heat treatment at acute heat stress, but differences in gene expression for indicator genes between individuals only become apparent during recovery. Our results underline the importance of considering a recovery phase in ecological stress experiments. Additionally, they indicate that functional intra-population variation in traits important for thermal tolerance exists in *Z. marina* and may have consequences for the persistence of eelgrass populations as well as their attached ecosystems in the face of ongoing decline due to global climate change.

Introduction

A pivotal question in ecology and evolution is how organisms react to stress imposed by global climate change (Etterson & Shaw 2001; Hoffmann & Sgro ; Lubchenco 1998; Root et al. 2003). Global climate change is accompanied by an

increase in mean variables, but also climate extremes such as droughts, floodings and heat waves is predicted to become more frequent (Easterling et al. 2000). Confronted with environmental stress, populations face three options to persist. Individuals can respond through plastic behaviour, migrate to environments they are already adapted, or, at the population level, there may be evolutionary adaptation to the new environmental conditions (Jackson & Overpeck 2000). While range shifts have been suggested to be a major biological effects of gradual changes in the environment (e.g. Parmesan 2006), migration as a reaction to typically very sudden extreme events in climate is likely to be difficult for individuals. Within a limited range, individuals may respond plastic, but adaptation is only possible on the population level through natural selection resulting in local rapid adaptation.

At the same time, it is of fundamental importance to elucidate the processes and mechanisms of evolution that allow adaptation to novel and increasingly complex selection pressures opposed by global climate change (Hoffmann & Sgro 2011). In the early 1950s, Fisher & Ford (1947) and Cain & Sheppard (1954) have provided seminal insights into the mechanisms and the potential speed of microevolutionary processes based on intraspecific variation. The importance of such variation to ecological processes has achieved considerable attention during the past decades, recently leading to an increased demand to fill existing knowledge gaps (Bolnick et al. 2011; Violle et al. 2012, in press). It has been shown that high levels of additive genetic variance within natural populations enable them to follow changing trait optima (Burger 1999) and may thus improve the potential to face and adapt to environmental changes (Jump et al. 2008). However, at present, we know remarkably little about the diversity of traits within populations that may become important under accelerating global change (Jump & Penuelas 2005; Rice & Emery 2003), such as variation in the timing of reproduction as well as tolerances to increased levels of solar radiation, higher aridity and temperatures (Jump & Penuelas 2005).

Assessing the potential for adaptation to global climate change is especially important for ecosystem engineering species as the persistence of a whole ecosystem community is depending upon the performance and survival of one or few single species (Ellison et al. 2005; Jones et al. 1994). Coastal marine habitats in temperate waters are mainly structured by kelp beds, salt marshes and seagrass meadows (Orth et al. 2006) and the ecosystem services provided by seagrass meadows in shallow littoral waters belong to the highest per unit area (Costanza et al. 1997).

Concurrently, seagrasses and adjusted ecosystems are of rising conservation concern due to accelerating global decline (Hughes et al. 2009b; Waycott et al. 2009). These declines result from various environmental, biological and extreme climatologic stressors (Orth et al. 2006). Population declines and physiological damages of elevated temperature on the seagrass Zostera marina have been described in the field (Greve et al. 2003; Reusch et al. 2005; Williams 2001) and in stress experiments (Bergmann et al. 2010). An open question which we discuss in this paper is whether there is within-population variation in thermal tolerance that may allow local populations that rarely face temperatures above 26°C (Bergmann et al. 2010) to track the environmental change via adaptive evolution.

Here we address the potential for in situ, adaptive evolution in a sessile, ecosystem foundation organism, the seagrass Z. marina. We assessed the heat stress reaction of five different genotypes of a northern Z. marina population during a realistic heat wave scenario of three weeks and a succeeding recovery phase of three weeks. Temperature profiles followed values measured in the coastal zone of the southwestern Baltic during the summer heat wave 2003 (Reusch et al. 2005). We looked at optimal quantum yield, leaf growth and shoot count as well as at gene expression data of three previously identified heat stress indicator genes. While important previous studies have focused on morphological and physiological variation among seagrass genotypes (Hughes et al. 2009a), to our knowledge this is the first study on a marine foundation species to assess the variation in heat stress gene expression among replicated individuals of one population as an answer to a realistic heat stress scenario in a common stress garden.

Materials and Methods

Study species

Our study species is eelgrass (Z. marina), the most widespread and locally abundant flowering plant along soft-sediment coasts of the northern hemisphere (den Hartog 1970). Eelgrass belongs to the seagrasses, a polyphyletic group of angiosperms that have reinvaded the marine environment from freshwater habitats (Les et al. 1997) and still possess the reproduction mode of their terrestrial ancestors. They reproduce clonally by building new shoots that grow out of rhizomes and

sexually by subaqueous flowering and pollination. The clonal reproduction mode makes eelgrass an ideal study organism in community and ecosystem genetics research (Whitham et al. 2006), as, by comparing replicated clones in a common garden environment, effects of genetic variation among individuals can be uncovered.

Experimental set up

Zostera marina ramets were collected in Doverodde (Denmark; North Sea) (N 56° 43.070' E 008° 28.446') April 8th 2010 in a water depth of 1.5 to 2m and water temperature of 11 °C. To achieve as many shoots as possible sharing the same genetic identity, we chose to sample seagrass material from small circular, obviously newly founded patches seaside of the meadow. 15 different patches were chosen to obtain material from various genotypes. Special care was taken to leave the rhizome connections intact. Seagrass ramets were taken to the AQUATRON Münster, a facility for seagrass culture containing 12 mesocosms (101cm x 120cm x 86.5cm) coupled in two closed seawater circuits with a flow rate of 1200l/h and planted within 48h after uprooting. Each tank housed 2 boxes (36,5 cm x 26,5 cm) that had been filled with natural sediment from the Baltic Sea, Falckenstein (N 54° 24.367 E 010° 11.438) to a height of ~ 10 cm. Ramets were tagged according to their patch of origin and planted such that each box housed seagrass material from approximately 7 donor patches, each representing a different genotype. Genotypic identity of each ramet was confirmed using microsatellite genotyping (see below). To prevent excessive growth of epiphytic algae, each tank housed ~50 individuals of periwinkles (*Littorina littorea*).

Water temperature was kept at field temperature for 20 days and then increased to 19°C in steps of 0.5°C/day. This temperature was kept for an acclimation period of 10 days. Illumination with artificial light (two 400W bulbs (Philips Master Green Power T 2000 K, 745µmol/s; Philips Master HPI-T PLUS 4000 K, 532µmol/s) per tank) in a 13h/11h light-dark cycle provided ~200µmol photons /m2/s at the surface of the leaves. To achieve nutrient values resembling those in temperate coastal waters of approximately 40µMol N and 3µMol P we fertilized the water during the heat wave. Fertilization was stopped to resemble nutrient conditions in summer periods.

Experimental treatments

On 26th of May 2010 temperature treatment was started by increasing temperature in half of the tanks from 19°C to 25°C with temperature steps of maximally 1°C/day. After having reached 25°C, a constant temperature was kept for 20 days to resemble the 2003 summer heat wave situation in the Baltic Sea (Reusch et al. 2005). Temperature was decreased afterwards to 19°C in steps of maximally 1°C/day and then kept constant at 19°C for four weeks to assess recovery data. Control treatment ranged between 18 and 19°C throughout the entire experiment (fig. 1). To achieve similar water chemistry in control and heat treatment, the two water flumes were constantly connected with an exchange rate of 1200l/hr.

Eighteen ramets (nine in each experimental treatment) of five different genotypes were chosen to be monitored throughout the experiment, leading to a total of 90 experimental shoots. We focussed on three fitness-correlated traits: leaf growth, vegetative shoot number and optimal quantum yield, which describes the percentage of light used by photosynthesis during darkness.

Growth rates were measured weekly by assessing the distance from leaf tip to meristem of the three youngest leaves of each experimental shoot across a 48 h interval. Each week photosynthetic activity was assessed with a PAM-2000 chlorophyll fluorometer (Walz) equipped with a leaf distance clip between 20.30 h and 22 h (30 minutes after onset of darkness). Optimal quantum yield, defined as the ratio of variable (Fv) to maximal fluorescence (Fm) of the dark acclimated sample was obtained following Schreiber et al. (1986). Fv is calculated by subtracting the initial fluorescence (F0) (all PSII reactions centers are active or “open”) from the maximal fluorescence (Fm) (all reaction centers of PSII are “closed”). Every second week all shoots in one box were counted. Physiological parameters were monitored throughout the experiment.

Genotyping, RNA extraction and cDNA preparation

Using four polymorphic microsatellites (GenBank Accession numbers: AJ009898, 009900, 249305, 249307) (Reusch 2000; Reusch et al. 1999) we genotyped all collected ramets to confirm genetic identity and to select experimental shoots. DNA samples were obtained from each individual shoot before planting by cutting off

2cm of the tip of a leaf. DNA extraction, genotyping and sequencing performed on an ABI 3100 Capillary Sequencer followed standard protocols, with the modification that we used Phire polymerase (Finnzymes, Finland).

RNA samples were taken between 8 and 10 am on days 22 (T3), 36 (T5) and 63 (T9) of the experiment (fig. 2). RNA samples were obtained from experimental shoots by cutting off 2 cm of the tip of the youngest leaf. Leaf blades were wiped clean and dipped into liquid N2 before immediate RNA extraction. RNA extraction was performed with the Invisorb RNA plant HTS 96 extraction kit (Invitek, Berlin, Germany) and followed standard protocols with the modification that sample disruption was done successively in groups of 32. We used the Quantitect Kit (Qiagen, Hilden, Germany) for reverse transcription and followed standard protocols. Out of a total of 9 samples per treatment, genotype and time point, five samples of five different genotypes were randomly used for expression analysis.

Quantitative PCR

We aimed at assessing genotypic variation in the expression response to an artificial heat wave in three indicator genes for heat stress, namely Hsp70, Hsp80 and Bip. These genes have been identified as contributing most to expression profile differences under different temperature treatments in Bergmann et al. (2010). The housekeeping gene we chose was the eukaryotic initiation factor 4A that has been shown to have a stable expression under different temperature regimes (Ransbotyn & Reusch 2006).

The amount of mRNA was assessed using quantitative real-time PCR (qPCR) on a StepOnePlus Cycler (Applied Biosystems, USA) with the Fast SYBR Green qPCR Master Mix (Applied Biosystems). PCR reactions, thermocycling details and calculation of amplification efficiencies followed Bergmann et al. (2010). All efficiencies were > 1.90, all R^2 were > 0.95.

All samples were triplicated on different plates. Relative expression values were calculated as:

$-\Delta CT = CT\ (housekeeping\ gene) - CT\ (target\ gene)$ (I)

$-\Delta\Delta CT = -\Delta CT(treatment) - (-\Delta CT(control))$ (II).

Data analysis

Normality assumptions for all response variables were assessed graphically, transformations and tests were performed accordingly using R (2009). Growth rates were summed over all growing leaves and standardized to 24h. To account for the fact that growth is correlated with shoot size, we performed a linear model with 'growth per 24h' and 'shoot size' and used the residuals of this model for further statistics. To account for repeated measures over time, a linear mixed-effects model was used to analyze growth rates, shoot count and optimal quantum yield with the factors 'temperature', 'time point', 'genotype' and the random effect 'shoot identity'.

To assess the variation in gene expression of the control over time, we performed a MANOVA on the –ΔCT values of only the control values for all three genes with the factors 'genotype' and 'timepoint' ($F_{3,71}$=5,00, p<0.01; $F_{3,71}$=6,78, p<0.001 and $F_{3,71}$=0,62, p=0,60 for genotype, timepoint and their interaction respectively). To account for the fact that we repeatedly measured the same experimental shoots over time, we performed MANOVA models for each gene separately, binding the –ΔCT -values of the different time points and applied Bonferroni correction for multiple testing. For model simplification and to account for changing control values, we decided to further work with relative expression values following the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). -ΔΔCT values were obtained by subtracting the replicated -ΔCTs of the treatment from the mean over all control -ΔCT-values for the control for each time point and genotype combination. To permit analyses of very low and very high expression values in the same statistic approach, relative expression data ($2^{-\Delta\Delta CT}$) were fourth root transformed. To assess differences in the combined expression pattern of the three indicator genes for different genotypes at different time points, an analysis of similarity (ANOSIM) was performed with the software Primer v6 (Clarke & Gorley 2006) based on the Bray-Curtis matrix.

Results

Physiological parameters

Contrary to our expectations, leaf growth rates, vegetative shoot count and optimal quantum yield were not significantly affected by the heat stress treatment (table 1). However, we found that genotypes grew differently in the two treatments and over time, reflected in significant genotype x treatment and genotype x time point interactions (table 1, fig. 1).

Looking at each time point separately, we found that the "genotype" x "treatment" interaction in the full model is mainly driven by a marginally significant "genotype" x "treatment" interaction at acute heat stress (T3) (table 2).

Shoots grew about 1,24 cm/day (+- 0.05cm SE) in our experiment and Optimal quantum yield ranged around 0,71 (+-0.001 SE).These values have previously been measured on healthy *Zostera* individuals in the lab and in the field (Bergmann *et al.* 2010; Macinnis-Ng & Ralph 2003; Silva & Santos 2004).

Table 1 Linear mixed effects models assessing the effects of heat stress treatment, genotypic identity and time point on leaf growth rates, shoot count and optimal quantum yield. numDF: degrees of freedom for numerator; denDF: degrees of freedom for denominator.

	numDF	denDF	F	P
Growth rates (corrected for size)				
Genotype (G)	4	80	0.53	0.71
Treatment (T)	1	80	0.39	0.53
Time point (TP)	3	240	19.02	**<0.0001**
G x T	4	80	2.68	**0.04**
G x TP	12	240	2.28	**0.02**
T x TP	3	240	1.72	0.16
G x T x TP	12	240	1.04	0.41
Shoot count				
Genotype (G)	1	78	4.7	**0.03**
Treatment (T)	1	78	1.44	0.23
Time point (TP)	1	324	0.41	0.52
G x T	1	78	0.3	0.59
G x TP	1	324	0.64	0.43
T x TP	1	324	1.9	0.17
G x T x TP	1	324	0.04	0.84
Optimal Quantum Yield				
Genotype (G)	1	86	0.08	0.78
Treatment (T)	1	86	0.01	0.94
Time point (TP)	1	266	5.84	**0.02**
G x T	1	86	0.58	0.45
G x TP	1	266	0.58	0.45
T x TP	1	266	0.37	0.54
G x T x TP	1	266	0.51	0.47

Table 2 ANOVAS assessing the effects of heat stress treatment, genotypic identity and time point on leaf growth rates at each measuring time point (T3 –T9) of the experiment. SS, sums of squares; DF, degrees of freedom

	SS	**DF**	**F**	**P**
T3				
Genotype (G)	9.63	4	3.12	**0.019**
Treatment (T)	0.04	1	0.06	0.81
G x T	7.01	4	2.29	**0.067**
Residuals	61.78	80		
T5				
Genotype (G)	2.56	4	1.19	0.32
Treatment (T)	1.39	1	2.6	0.11
G x T	2.62	4	1.22	0.31
Residuals	42.83	80		
T7				
Genotype (G)	2.58	4	0.86	0.49
Treatment (T)	1.84	1	2.47	0.12
G x T	0.22	4	0.075	0.99
Residuals	59.65	80		
T9				
Genotype (G)	7.1	4	1.52	0.21
Treatment (T)	1.19	1	1.01	0.32
G x T	8.81	4	1.88	0.12
Residuals	93.61	80		

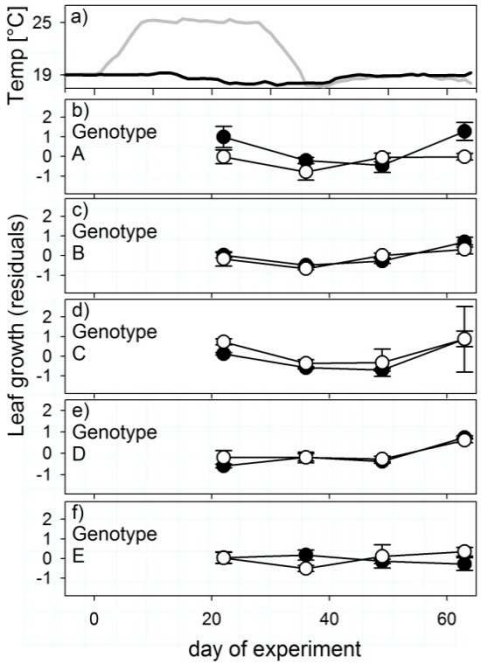

Fig.1 Time course of temperature and leaf growth corrected for size (residuals) in *Z. marina* (eelgrass). Panel (a) depicts experimental temperatures (grey: heat stress treatment; black: control tanks).Panels (b)–(f) give mean leaf growth +- SE corrected for size (residuals) of shoots for genotypes A-E, respectively.

Gene expression

The gene expression of all three genes differed significantly between control and heat-treatment (MANOVAs on –ΔCT-values, table 3).

Interestingly, while during heat stress (T3) and long term recovery (T9) genes were upregulated in the treatment compared to the control (leading to positive -ΔΔCT values) we also found downregulation of the stress associated genes during short term recovery (T5) (fig. 2).

Time point specificity of expression profiles

As we were interested in resolving differences in the expression profiles during the time course of the experiment, we averaged -ΔΔCT values over genotypes. We found significant differences between the theoretical control (T0; -ΔΔCT=0), heat stress expression profile (T3), recovery profile (T5) and long-term recovery profile (T9). Pair wise tests revealed that all combinations were highly significantly different from each other. Interestingly, the MDS plot shows a marked difference between both recovery expression profiles (T5 &T9) and the heat stress expression profile (T3) (see fig. 3).

Table 3 Gene expression in the seagrass *Z. marina*; MANOVA significance values for differences between genotypes and treatment (-ΔCT values for single time points are combined) for single genes; significant differences are highlighted in bold. d.f., degrees of freedom; MS, mean squares.

	d.f.	**MS**	**F**	**P**
Bip				
Genotype(G)	4	0.78	3.494	**<0.001**
Treatment(T)	1	0.8	51.87	**<0.001**
G x T	4	0.24	0.89	0.56
Residuals	40			
Hsp70				
G	4	0.75	3.35	**<0.001**
T	1	0.63	21.44	**<0.001**
G x T	4	0.41	1.6	0.1
Residuals	40			
Hsp80				
G	4	0.74	3.25	**<0.001**
T	1	0.57	16.57	**<0.001**
G x T	4	0.39	1.48	0.14
Residuals	40			

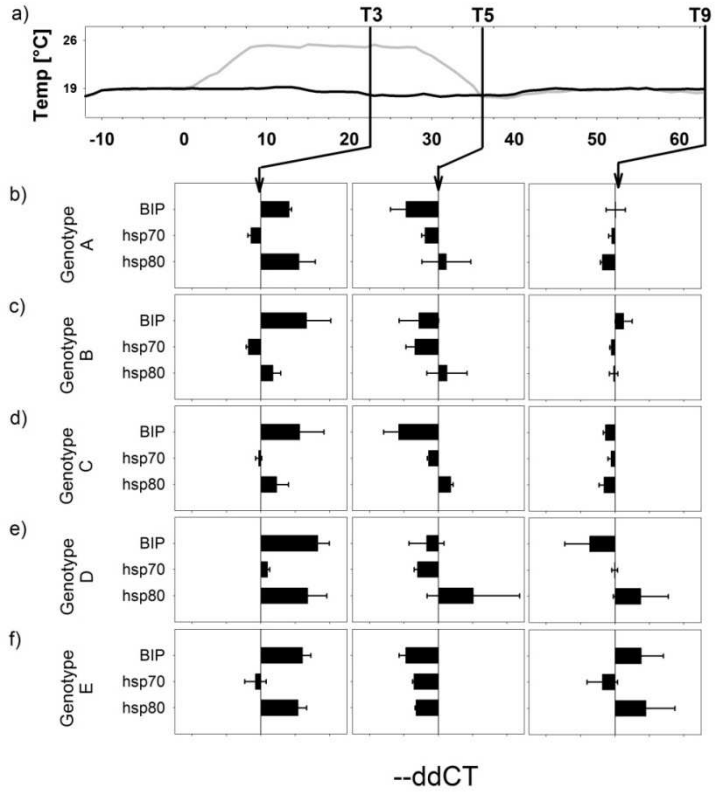

Fig. 2 Relative gene expression in the seagrass *Z. marina*; -ΔΔCT values of 3 stress associated genes that were induced relatively to the control treatment. For comparison, the temperature course of the experiment is given in panel (a) (grey: heat treatment, black: control) and RNA sampling time points T3–T9. Panels (b)–(d) depict mean -ΔΔCT values ± 1 SE (n = 5) for Genotypes A to E. Gene expression was quantified during the simulated heat wave (T3), directly after the heat wave (T5) and after 3 weeks of recovery (T9).

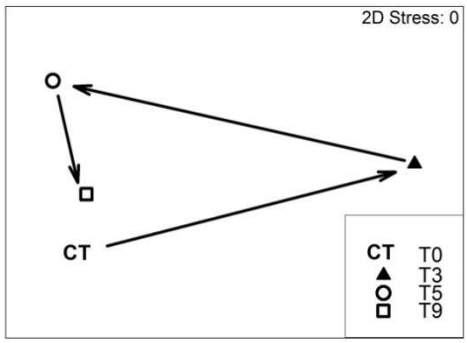

Fig. 3 Gene expression in the seagrass *Z. marina* during and after a heat wave. Multi-dimensional scaling (MDS) after ANOSIM analyses based on mean -ΔΔCT-values averaged over genotypes. Each time point is represented by a x–y-coordinate, with the distance to each other proportional to the similarity of the expression profile. Arrows depict the time course of the experiment.

Genotype specificity of expression profiles

To find out if genotypes reacted differently to the heat wave in terms of gene expression we looked separately at the expression profiles of each single time point (ANOSIM analyses on -ΔΔCT values). While genotypes responded equally to the heat wave, expression profiles were significantly different when temperatures returned to control conditions (short term recovery T5) with this difference lasting until four weeks of recovery (long term recovery T9) (table 4, fig. 4).

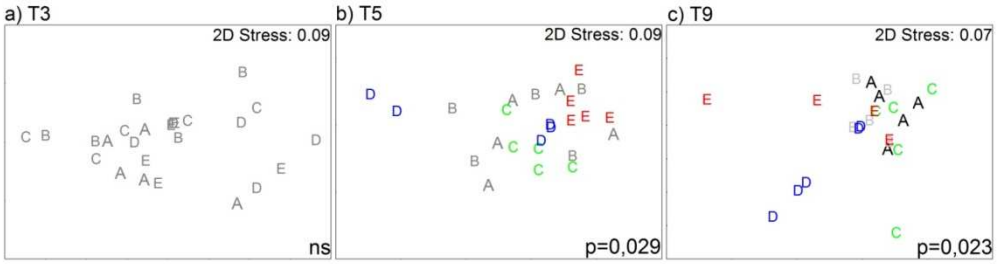

Fig. 4 Genotype-specificity of heat stress gene expression over time in the seagrass *Z. marina*; MDS based on relative gene expression (-ΔΔCT) for time points T3 (a) to T9 (c). For the position of time points refer to Fig x. The significance values of the underlying ANOSIM analysis are given, testing the null hypothesis of no gene expression difference among genotypes at single time points. Each letter represents the gene expression vector of 3 genes as an x–y-coordinate of one single shoot, with the distance to each other proportional to the similarity of the expression profile. A, genotype A; B, genotype B; C, genotype C; D, genotype D, E, genotype E; different colors represent genotypes that are significantly different from other genotypes (see table 4).

Table 4 Pair-wise comparison of relative gene expression (-ΔΔCT) in the seagrass *Z. marina* among five different genotypes at three time points during and after an experimental heat wave using ANOSIM. Global R and significance values for single time points (T3, T5, T9) and significance values for pair wise genotype comparisons at each time point are given; Significant differences are highlighted in bold; A, genotype A; B, genotype B; C, genotype C; D, genotype D, E, genotype E.

	Global R	**Global p**	**Pair wise tests**	**B**	**C**	**D**	**E**
T3	0.028	0.3	**A**	0.17	0.21	0.11	0.41
			B		0.8	0.14	0.28
			C			0.33	0.6
			D				0.56
T5	0.15	**0.0029**	**A**	1	0.36	0.46	0.14
			B		0.14	0.63	0.11
			C			0.079	**0.008**
			D				**0.008**
T9	0.12	**0.0023**	**A**	0.56	0.79	**0.048**	0.13
			B		0.15	0.12	0.31
			C			**0.032**	**0.04**
			D				0.25

Discussion

Our approach allows gaining insight into the diversity of reactions to heat stress among different individuals within a *Z. marina* population. Looking at the physiological parameters, we found that at acute heat stress individuals differ in their leaf growth in different treatments. Several studies have assumed the cellular stress response to negatively influence regular cell functions by toxic effects of accumulating Hsp-congeners as well as overexploitation of energy resources and blockage of cell components that are involved in protein syntheses and catabolism (reveiwed in Feder & Hofmann 1999). Conceivably, an appropriate heat stress reaction comes with a cost - resources invested in gene expression of stress related genes cannot be invested in leaf growth any more.

While differing in their growth rates over treatments, all genotypes show the same stress expression profile for the indicator genes at acute heat stress. Differences in gene expression between individual genotypes do not become apparent until recovery, when reactions of individuals diverge significantly. This is in line with the divergence at the population level in an earlier study (Franssen et al. 2011b) in which we compared the expression answer to a heat wave of 25°C of *Z. marina* from different populations originating from contrasting thermal regimes. We found minor differences during acute heat stress between whole transcriptomes of different populations assessed with next generation sequencing (Franssen et al. 2011b). Differences at acute heat stress existed in only a few of several genes annotated as classical Hsps or with the functional term “stress abiotic heat”, among them two genes that had been identified as heat stress indicator genes with a qPCR candidate gene approach in a previous study (Bergmann et al. 2010). In contrast, profiles of the whole transcriptome diverged considerable for populations during recovery (Franssen et al. 2011b). Acute heat stress seems to limit the individual reactions - a defined expression pattern of Hsp-expression is “required” to be able to cope with acute heat stress. At stress relief and during recovery different variants of coping are “allowed”, as stress is not acute anymore and different genotypes show their own typical recovery expression profile.

The present study differs from the outcomes of the previous experiment in terms of shoot reduction. While in Bergmann et al. (2010) we found a significant reduction in shoot count for different populations irrespective of their thermal pre-

adaptation, we did not find a decrease in vegetative shoot count in the recent study. We assume this difference mainly to be reasoned in different sampling procedures: For the present experiment we collected ramets from random patches within the seagrass meadow, as we then were primarily aiming to gain representative genotypic diversity in each experimental unit. In contrast, for the previous experiment we were aiming to have replicates of each genotype in the experimental units. We focused on sampling “young” circular seagrass patches seaside of the meadow composed of only one genet for which we easily could follow rhizome connections. These genotypes, in contrast to genotypes situated within the tight rhizome mat in a dense meadow have successfully pioneered formerly uninhabitated sediment. We think that this ability can certainly lead to a different performance under stress.

After environmental challenges like extreme events in climate, responses on the cellular level are induced to circumvent long-term damage consequences. A very first protection mechanism is the expression of heat shock proteins that function as molecular chaperones and protect other proteins from degradation (reviewed in Sorensen et al. 2003). This makes Hsp-induction a fundamental repertoire for surviving in a fluctuating environment (reviewed in Feder & Hofmann 1999) and a crucial mechanism for acclimation at the cellular level especially for sessile organisms (Wang et al. 2004).

Although the very immediate answer to heat stress is very conserved (e.g. Gupta 1995) - reflected by a common acute heat stress expression pattern for all genotypes in our experiment – expression levels of Hsps have been shown to be under selection and the amount, congener composition and thresholds for expression of Hsps have been identified to be closely related to the habitat of the species or subpopulation (reviewed in Reusch & Wood 2007; Sorensen et al. 2003). Natural selection acting on the efficiency of the recovery gene expression profile becomes particularly important when assessing the potential for a population to face and endure prospected climate change. Marine organisms are adapted to a life in water, which through its high specific heat capacity (Steele 1985) is a by far more stable temperature environment than the terrestrial environment (Feder & Hofmann 1999). This capacity first leads to a dampening of extreme temperature fluctuations, but if critical temperature values are reached, they typically are also kept much longer, making aquatic organisms particularly susceptible to climate extremes in temperature (Reusch & Wood 2007). Particularly for sessile aquatic organisms the potential to face

and recover from sudden heat stress events that they cannot avoid by means of migration should be of major importance (Feder & Hofmann 1999). Crucial for enduring heat stress is not only the immediate answer at acute heat stress, but also resilience and recovery after the acute stress event. We found this answer to be genotype-specific in our experiment and argue that natural selection should act strongly on this variation.

Biodiversity on all scales is generally considered a key prerequisite for stability and functioning in biological systems. Biodiversity and species richness are important factors for productivity, resistance and resilience of ecosystems (reviewed in Hooper *et al.* 2005). In communities with few species as is the case for seagrass systems along the coasts of Northern Europe with very low seagrass diversity, genotypic diversity can replace the function of biodiversity and enhance ecosystem resilience (e.g. Hughes & Stachowicz 2004; Reusch & Hughes 2006). In the face of rapid environmental change, the term 'option value' of genetic diversity has been introduced. Even if the biggest proportion of genetic variants within a population may not be adaptive, the fraction that potentially is adaptive should be proportional to the amount of the initial genetic diversity within the population (reviewed in Jump *et al.* 2009).

A seminal study conducted by Hughes et al. (2009a) assessed clonal variation of different eelgrass clones that were common gardened over 10 weeks after an acclimation period of 2 years. While they found considerable morphological and physiological variation among eelgrass genotypes, leaf growth was the only physiological parameter we identified to vary among genotypes over treatments and the course of the experiment in our study.

However, our results indicate that different individuals are not similar in their recovery from heat stress, which is in line with a previous field study (Reusch et al. 2005). We emphasize the necessity for ecological experiments with realistic stress scenarios including recovery phases and endorse the increasing demand for the acknowledgement of the importance of intraspecific variability in ecology (reviewed in Violle et al. 2012, in press). In contrast to the variation among populations, the variance in reaction to heat stress among individuals was only apparent during recovery and we would have missed it completely only looking at the acute heat wave reaction of individuals. However, to assess the potential for adaption of a population it is important to look at the variation on the level of single individuals as this is the

repertoire natural selection is working on. In the face of ongoing population decline functional intra-population variation in key traits for responses to climatic changes has to be considered carefully (Jump & Penuelas 2005), especially for ecosystem engineering species that influence the fate of whole ecosystems. Without neglecting variation between populations, future work should include standing as well as functional genotypic variation with emphasis on the inter-individual level within populations to better predict their potential for microevolution in the face of global climate change.

Acknowledgements

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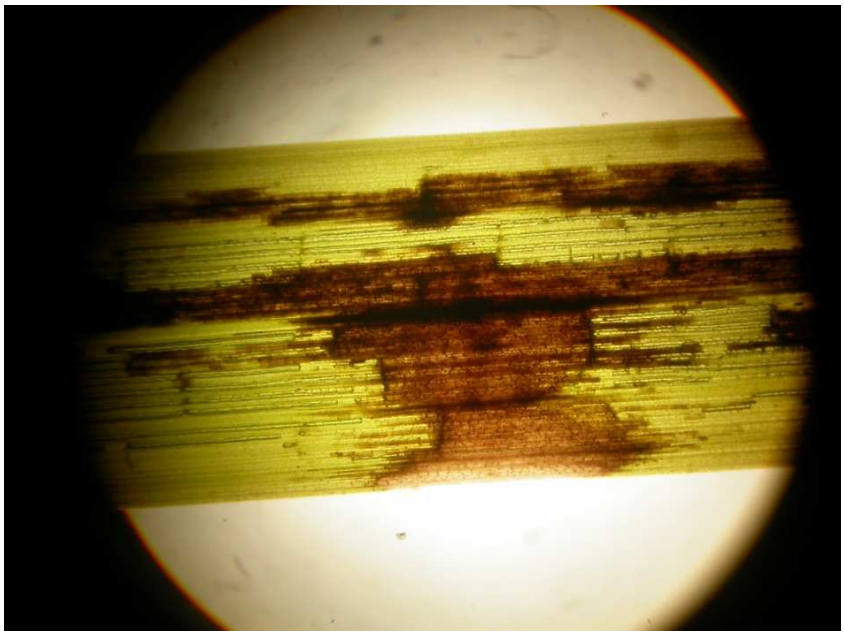
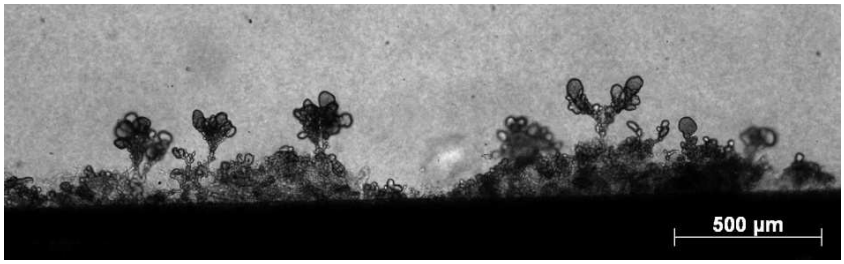
Chapter IV



Eelgrass leaf with *Labyrinthula* infection
(picture by Birgit Fricke)



The beauty lies in the eye of the beholder -
Infection from a different perspective:
Labyrinthula growing out of a *Z.marina* leaf
(picture by Birgit Fricke)

Chapter IV

A quantitative real-time PCR assay for the seagrass pathogen *Labyrinthula zosterae*

Abstract

The protist *Labyrinthula zosterae* (Phylum Bigyra, sensu Tsui *et al.* 2009) has been identified as a causative agent of wasting disease in eelgrass (*Zostera marina*), of which the most intense outbreak led to the destruction of 90% of eelgrass beds in eastern North America and western Europe in the 1930s. Outbreaks still occur today, albeit at a smaller scale. Traditionally, *L. zosterae* has been quantified by measuring the necrotic area of *Z. marina* leaf tissue. This indirect method can however only lead to a very rough estimate of pathogen load. Here, we present a quantitative real-time PCR (qPCR) approach to directly detect and quantify *L. zosterae* in eelgrass tissue. Based on the internal transcribed spacer (ITS) sequences of rRNA genes, species-specific primers were designed. Using our qPCR, we were able to quantify accurately and specifically *L. zosterae* load both from culture and eelgrass leaves using material from Europe and North America. Our detection limit was less than one *L. zosterae* cell. Our results demonstrate the potential of this qPCR assay to provide rapid, accurate and sensitive molecular identification and quantification of *L. zosterae.* In view of declining seagrass populations worldwide, this method will provide a valuable tool for seagrass ecologists and conservation projects.

Introduction

The pandemic decline of *Zostera marina* due to the wasting disease in the 1930s is regarded as one of the most serious events for seagrass population biology (Milne & Milne 1951). Over 90% of eelgrass populations in Europe and North America disappeared within a very short time span (e.g. Cottam 1934; Short et al.1987). As *Zostera marina* is a major ecosystem engineering species sensu Jones et al. (1994), its decline also resulted in decreases of associated animal populations (Milne & Milne 1951; Rasmussen 1977) as well as complete elimination of a species (Carlton et al.

1991). Moreover, erosion of sediments on coastlines was reported, caused by a reduction in sediment solidification capacity in regions with eelgrass declines (Christiansen et al. 1981; Rasmussen 1977; Wilson 1949). Due to the severe ecological and economical consequences that impacted many services associated with the seagrass ecosystem (Costanza et al. 1997), eelgrass wasting disease is considered one of the major epidemic diseases in marine ecosystems (Muehlstein 1989).

Labyrinthula zosterae (Muehlstein et al. 1991) is a protist belonging to the group of stramenopiles that develops in the leaves of *Z. marina* and was identified as a causative agent of eelgrass wasting disease (Den Hartog 1987; Muehlstein 1989, 1992; Muehlstein et al. 1988; Short et al.1987). *Labyrinthula zosterae* and its congenerics, together with aplanochytrids and thraustochytrids, form the class Labyrinthulomycetes (*sensu* Kirk et al. 2001; Tsui et al. 2009). *Labyrinthula* spp. form extracellular networks for communication, nutrient transport and locomotion (Preston & King 2005). They are commonly associated with seagrasses and macroalgae, and more recently with terrestrial plants (Douhan et al. 2009; Olsen 2007).

Labyrinthula zosterae mainly occur within leaf parenchyma cells, where they damage chloroplasts leading to reduced photosynthetic activity, discoloration and development of expanding brown or black necrotic spots finally resulting in leaf loss (Renn 1936, Raghukumar 2002, Ralph & Short 2002). A “Wasting Index”, based on quantification of leaf area covered by such lesions, has been developed as a measure of infection (Burdick et al. 1993, Hily et al. 2002). This approach does not allow identification and quantification of *L. zosterae* infection before the development of visual symptoms in infected eelgrass leaves, even though *L. zosterae* has also been found outside lesions (Renn 1936, unpublished data A.-C. Bockelmann and D. L. Martin). Furthermore, when using the “Wasting Index”, it is not always possible to distinguish between necrotic tissue caused by mechanical or other injuries and by *L. zosterae.* A direct quantification method that allows detection of invisible *L. zosterae* infections and the distinction from other necrosis-inducing processes is therefore of major importance for the study of the wasting disease.

Quantitative polymerase chain reaction (qPCR) based approaches have proven to allow fast detection and precise quantification of species and their abundance in various organisms (e.g. for fungi and corals respectively: Haugland et al. 2004; Mieog et al. 2007; Mieog et al. 2009). qPCR assays allow the analysis of large sample sizes

and can also be applied to dried material. The aim of this study was to establish a method of accurate detection and quantification of *L. zosterae* infection by establishing a qPCR assay based on a cosmopolitan collection of samples.

Material and methods

Labyrinthula cultures

For sequencing and qPCR establishment *Z. marina* samples from five locations on the European and US coast were used (Table 1). For the direct quantification of *Labyrinthula zosterae* from eelgrass leaves, samples were taken from five locations on the NW-European coast (Wackerballig, Flensburg Fjord, Germany; Lemvig, Limfjord, Danmark; Sandspollen, Oslo Fjord, Norway; Svartholm, Archipelago Sea, Finland; Sylt, North Sea, Germany). In order to obtain *L. zosterae* cells free of any host DNA, *L. zosterae* from leaf samples were cultivated on a seawater agar according to a protocol modified after Muehlstein et al. (1988).

For one liter of seawater-agar medium (for 100 Petri-dishes ø10 cm): 12 g agar (Agar UltraPure, USB Corporation, USA), 1 g glucose (Roth, Germany), 0.1 g yeast extract (Roth, Germany), 0.1 g peptone (Fluka, Germany) and 1 L Milli-Q water were mixed and autoclaved 20 min at 121 °C, and 25 g Instant Ocean artificial sea salt (Instant Ocean, Spectrum Brands, USA) added while still hot (salinity: 25 psu). After cooling to 50 °C, 25 mL Penicillin-Streptomycin (MP Biomedicals, USA) and 10 mL horse serum (Invitrogen, USA) were added, mixed, and the medium poured immediately.

Leaf samples (1-3 cm) of the leaves showing visual symptoms of wasting disease were dipped in 0.5% hypochlorite solution in seawater for 20 s of surface sterilization, rinsed with Milli-Q water for 10 s and soaked in artificial seawater for 1 min. Washed leaf samples were separately placed on culture plates and incubated at 25 °C in a climate cabinet. Cultures were checked after three, five and eight days for growing *L. zosterae*. After 2-4 weeks cultures were transferred to new agar plates.

Species identification

In the five cultures used here to develop the assay (Table 1), we identified a 1200 bp region of the 18S rRNA gene using direct Sanger sequencing of an amplicon produced by the 18S f- and r- universal primers proposed by Medlin et al. (1988). Based on initial sequencing and alignment with other published Labyrinthulid sequences, three novel sequencing primers (18S_f2: 5'- CGA ATG TAG CGT TTA CTG TG-3'; 18S_r2: 5'- CCG TCA ATT CCT TTA AGT TTC AGC-3', 18S_r3: 5'- GTG CCC TTC CGT CAA TTC C-3') were designed within conserved portions of the 18S rDNA gene to enable the contiguous determination of the entire amplicon.

The consensus sequence was manually edited and aligned in CodonCode aligner (v3.7.1, CodonCode Cooperation, USA). All five sequences were blasted against the non-redundant nucleotide GenBank data base on NCBI and revealed 99% similarity to 2 partial 18S sequences designated as *L. zosterae* from Woods Hole and San Juan Island (GenBank accession numbers AF265334 and -5, respectively) by Leander & Porter (2001) (E-value = 0.000). Table 1 shows the GenBank accession numbers for the 18S sequences of the five *L. zosterae* samples.

Cell count

To standardize the qPCR results with known cell numbers of *L. zosterae*, cell counts were performed for five cultures of different origin (Table 1) with a Fuchs Rosenthal counting cell chamber (small square area 0.0625 mm², depth 0.2 mm, volume 0.0125 µL). *L. zosterae* cells were scratched off an equally sized surface region of the agar and suspended in HPLC water. Two microliters of the cell suspension were loaded on the chamber. After sedimentation of the cells (1 min) the cell number in each of five randomly chosen small squares was counted twice at 160x magnification.

DNA extraction

DNA extractions were performed with an Invisorb spin tissue mini kit (Invitek, Berlin, Germany) using standard procedures for extractions of pure *L. zosterae* DNA to establish a standard curve for the qPCR. For calibrating extraction efficiency, and quantification of *L. zosterae* in situ, 1 µL of UltraPure™ salmon sperm DNA solution

(Invitrogen, life technologies, USA) (at 500 ng/µL) was added to each extraction to saturate silica columns with DNA, thereby improving recovery of small amounts of target DNA.

Table 1: *Labyrinthula zosterae* strains isolated from *Zostera marina* used for qPCR calibration of extraction efficiency

***Labyrinthula* strain ID**	**seagrass collection site**	**coordinates**	**ITS (acc. number)**	**18S (acc. number)**
1b	Washington USA	N48°45.64' W122°54.94'	JN121410	JN121404
12b	Virginia USA	N37°06.47' W75°58.08'	JN121409	JN121405
LA3	Falckenstein Germany	N54°24.37' E10°11.44'	JN121412	JN121408
LA47	Doverodde Denmark	N56°43.07' E08°28.45'	JN121411	JN121406
LA52	Gabicce Mare Italy	N43°57.97' E12°45.86'	JN121413	JN121407

ITS sequencing and Primer design

qPCR primers were designed for the internal transcribed spacer (ITS) region between the small subunit (SSU) and large subunit (LSU) of the rDNA (Gardes & Bruns 1993; Hillis & Dixon 1991). Amplification and sequencing of the ITS region of the five *L. zosterae* samples (Table 1) was done by cycle sequencing (Sanger et al. 1977) with the universal primers ITS1 and ITS4 (White et al. 1990) on a 3130xl capillary sequencer (Applied Biosystems, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

The ITS sequence of *L. zosterae* was compared to additional ITS sequences from Labyrinthulomycetes strains cultured from five different seagrass species: *Halodule wrightii*, *Phyllospadix scouleri*, *Ruppia maritima*, *Thalassia testudium* and *Posidonia oceanica* (unpublished sequences, D. L. Martin) and primers were manually designed specifically to amplify only the consensus ITS sequence of *L. zosterae* in Primer3 (Rozen & Skaletsky 2000). The ITS sequences of the additional seagrass species are not shown, as the exact species identification based on 18S sequences is not yet clarified (D. L. Martin and A. A. Boettcher, unpublished data).

The PCR reaction mix contained 2 µL 5x buffer, 1 µL Pre-mix, 0.5 µL of forward and reverse primer (5 pmol/µL), 5.5 µL of Aqua dest. and 1 µL of template. The

thermal cycling conditions were as follows: initial denaturation was performed for 4 min at 96 °C, followed by 35 cycles of 20 s at 96 °C, 10 s at 50 °C and 2 min at 60 °C. A consensus ITS sequence was obtained with ClustalW in BioEdit (v7.0.5) (Hall 1999) and edited manually. Primers were designed with the Primer3 program (Rozen & Skaletsky 2000). Table 1 shows the GenBank accession numbers for the ITS sequences of the five *L. zosterae* samples.

Optimization of the qPCR-protocol

qPCR was performed in a 20 µL final reaction volume on the StepOnePlus Cycler (Applied Biosystems, USA): 10 µL Fast SYBR Master Mix (Applied Biosystems, USA), 0.8 µL of forward and reverse primer (5 pmol/µL), 4.4 µL of HPLC water and 4 µL of sample. For the calibration of the extraction efficiency and the in situ quantification from leaf samples, 1:10 diluted samples were used. The thermal cycling conditions were as follows: initial denaturation was performed with 5 s at 98 °C and 20 s at 95 °C, followed by 45 cycles at 60 °C for 30 s and 95 °C for 5 s. A melting curve (65-90 °C) was added to confirm the identity of the product. All samples were tested in triplicate and the standard deviation of triplicates never exceeded 0.3 Ct.

To test whether the PCR reaction is independent of template concentration, standard curves of a dilution series with *L. zosterae* DNA extracted from four different strains were analyzed (details not shown). As all efficiencies were >0.85 and all R^2 were >0.99, PCR efficiency was independent of DNA concentration.

Calibration of extraction efficiency

After we performed a standard dilution series on DNA extracts of *L. zosterae*, we tested whether the extraction efficiency is consistent and proportional to *L. zosterae* cell number. Cell suspensions with known cell concentration (803.25-2128.75 cells per µL, same five *L. zosterae* strains as used before) were diluted thirteen times two-fold in a geometric series down to 1:16384. DNA was extracted from each dilution step. Salmon sperm DNA was added as described above. In addition to no-template controls, salmon sperm DNA was used as additional negative control to exclude cross contamination of individual samples.

Quantification of *Labyrinthula zosterae* in eelgrass leaves

As a first application of the quantification method for the *Z. marina* host, 10 leaves from five different *Z.marina* locations (see above) were analyzed; from five samples *L. zosterae* was successfully isolated, while from the other five samples isolation and cultivation of *L. zosterae* was unsuccessful. For the comparative analysis, leaves were longitudinally cut and one half dried for later DNA extraction while the other half served as the source for cultivation of live isolates on agar. Moreover, all successful isolates came from black lesions, while the unsuccessful ones came from healthy looking tissue. As above, crude DNA extracts were prepared using the Invitek tissue kit after grinding the dried leaf material for 30 s in a ball mill, and adding salmon sperm DNA to improve recovery from kit filters. Target DNA was purified using a one-step PCR inhibitor removal kit (Zymo Research, USA). As a template, 4 µL of a 1:10 diluted crude DNA extract was used. A standard of *L. zosterae* preparations containing 42, 1355 and 21683 cells per reaction was added for absolute quantification. Cell numbers per leaf were calculated from parameters of a linear regression of log-transformed cell counts against CT-values (x).

Results

Calibration of DNA extraction efficiency

The following primer pair was used for the qPCR assay: Lz2forward, 5'- CTA AGA CTA AAC GAG GCG AAA GCC TAC-3'and Lz2reverse, 5'- AGG TTT ACA AAA CAC ACT CGT CCA CA-3'(size of amplicon: 202 bp). With the help of a geometric dilution series, we analyzed whether the qPCR assay results are proportional to *L. zosterae* cell number used for extraction. Here, the mean extraction efficiency was 96.4% (s.d. = 6.94), all R^2 = 0.98 (s.d. = 0.01) (Fig. 1). At the highest dilution step (1:16384) reproducible CT values <39 could still be produced. This results in a minimum detection level of 0.08 ± 0.02 individual *L. zosterae*-cells (equivalent with $\log_2$ (3) = 1.58; Culture C133; figure 1e).

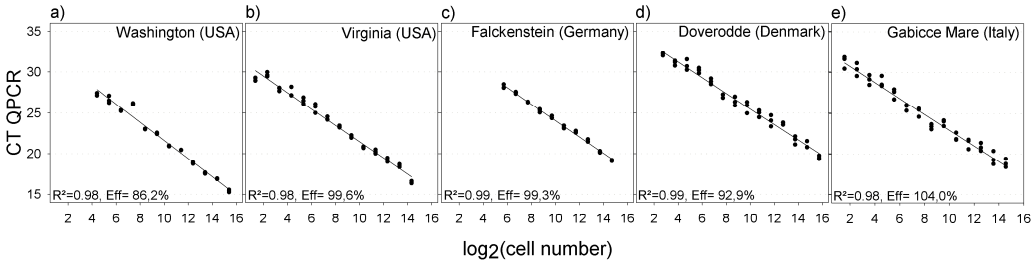


Fig. 1: Calibration of extraction efficiency using a cell dilution series. The number of *L. zosterae* cells ($\log_2$) is plotted against Ct values at which the qPCR reaction reaches the threshold of 0.2 fluorescence. Five different cultures were tested: a) Washington, USA (lab culture ID 1b); b) Virginia, USA (lab culture ID 12b); c) Falckenstein, Germany (lab culture ID LA3); d) Doverodde, Denmark (lab culture ID LA47); e) Gabicce Mare, Italy (lab culture ID LA52). R^2 and efficiencies are given in each individual figure; efficiencies are calculated from equation [1] $E= 10^{-1/\text{slope linear regression}} -1$.

Quantification in dried *Z. marina* leaf material

Quantification of *L. zosterae* cells in dried *Z. marina* leaf samples was successful. Four of the five *Z. marina* leaf samples, which had lesions and from which *L. zosterae* could be cultivated, gave positive QPCR results (CT range 26 - 31, cell numbers range 806 - 25812). The species identification of the fifth sample with visible lesions (isolated from Svartholm, Archipelago Sea, Finland) is analysed in the submitted manuscript of Bockelmann et al. (under revision in Marine Ecology Progress Series). For the samples where no *L. zosterae* could be cultivated, we could

detect low numbers in 4 out of 5 samples (< 7 cells); while in one sample, a moderate number of cells (58) was detected.

Discussion

A real-time qPCR technique for the detection and quantification of the seagrass pathogen *L. zosterae* was developed and validated. All five strains used as basis for qPCR development have highly similar 18S sequences to the published sequences designated as *L. zosterae* by Leander & Porter (2001); in addition, all five of these isolates used as basis for qPCR development have an identical ITS sequence. It is also noteworthy that isolates having this ITS type have proven to be virulent in numerous laboratory infection assays (A. A. Boettcher & D. L. Martin, unpublished data). Our method is culture-independent and detects *L. zosterae* within the eelgrass host. This way, information about the distribution and abundance of *L. zosterae* in declining eelgrass beds can be collected and can enhance our understanding of *L. zosterae* biology and its interactions with the host *Z. marina*. Especially important, even very low abundances of *L. zosterae* in asymptomatic samples can be measured by our assay.

High sensitivity or the ability to detect small quantities of the target pathogen DNA is a critical element in the development of a qPCR assay. The detection limit of the qPCR was at least 0.08 ± 0.02 cells per qPCR reaction, which is in line with the sensitivity of detection of pathogenic bacterial species (Jung et al. 2010; Lambertz et al. 2008).

Linear regressions for the five different strains (Fig. 1) had equal slopes (leading to equal extraction and PCR efficiencies) but different intercepts corresponding to different absolute cell counts at identical CT values for different strains. This variance most probably results from a combination of two sources of variance, the biological variance among the different strains and the variance of the initial cell counting process. To make data fully comparable, a standard sample with DNA extracts of defined numbers of *L. zosterae* cells should be run on each qPCR plate and CT values of all other samples of interest are related to the standard sample. The use of internal standards is a common approach in qPCR experiments (e.g. Niesters 2001). For gene expression assays the 2^{-ddCT} method (Livak & Schmittgen 2001) is often used. In this relative quantification approach the expression of a target gene is

measured relative to the gene expression of a housekeeping gene. For absolute quantification experiments, where the absolute copy number of the fragment of interest is required, the qPCR signal is commonly related to a standard curve. For quantification experiments with our assay, we recommend the use of the absolute standard curve approach.

In conclusion, the assay described here provides a major step forward in quantifying *L. zosterae* densities from dried *Z. marina*-tissue. This rapid, sensitive and specific method for the detection and quantification of *L. zosterae* can equally be applied to culture samples as well as to field samples of its host *Z. marina*.

Acknowledgements

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Conclusion

In summary, the results of my studies demonstrate intraspecific variation in the answer to heat stress in the seagrass *Z. marina* and provide a first step towards unravelling the genetic basis of thermal adaptation in eelgrass. While different populations suffered equally from shoot loss during an experimental heat wave, irrespective of their thermal pre-adaptation (Chapter I), individual genotypes varied in growth rates over treatments and over the course of the experiment (Chapter III). I found several eelgrass genotypes within one population (Chapter III) as well as different populations to vary significantly in stress gene expression (Chapters I) as a response to a simulated heat wave. Expression differences between populations in selected stress-associated genes were apparent at the onset of the heat wave (Chapter I). Notwithstanding, a subsequent global transcription profiling revealed that those effects were of relatively minor importance compared to massive differences in gene expression during the recovery phase between two of the populations (Chapter II). This is in line with findings on the genotype level within one population (Chapter III) which showed differences in the expression profiles of selected stress-associated genes between replicated individuals only in the recovery phase.

Two different approaches identified identical indicator genes for heat stress. For qPCR primer development (Chapter I) I chose genes that were related to “heat stress” and “heat shock” via a keyword search in the EST data base Dr. Zompo (Wissler *et al.* 2009). The database is comprised of tentative unigenes obtained by traditional Sanger sequencing and was thus relatively small compared to subsequent massive extensions that were achieved by next generation 454-sequencing. This target gene qPCR approach revealed Bip (a luminal binding protein associated with drought stress in other species (Valente et al. 2009)) and Hsp70 and Hsp80 (two classical Hsps) to be responsible, in combination, for over 50% of the expression variation between populations. The subsequent next generation sequencing approach (Chapter II) revealed expression differences in only a few of the genes annotated as classical Hsps or with the functional term “stress abiotic heat” between populations at acute heat stress, among them Bip and a gene annotated as Hsp81 (see Fig II-S5, Appendix). A BLAST search of the primer pairs used for qPCR assays in Chapter I against the contigs obtained in Chapter II showed that the tentative unigene annotated as Bip was identical in both approaches and that the primers that amplified

Hsp80 in Chapter I matched a contig that was annotated as Hsp81 in Chapter II (personal communication S.U. Franssen). Thus, the same indicator genes were detected with two different approaches. Nonetheless, with next generation sequencing now being commonly available as well as affordable, it seems advisable for future studies to either first systematically explore the overall transcription profile with next generation sequencing (Chapter II) and subsequently study selected indicator genes in a replicated QPCR approach or to directly use replication in the next generation sequencing approach. Such systematic approach may be particularly useful for studies investigating environmental stressors for which the genetic response is not as well understood as the heat stress response.

The assessment of gene expression in stress related indicator genes is particularly valuable in complex organisms with complex life cycles such as seagrasses, which combine vegetative and sexual reproduction, and have a physiological storage compartment, the rhizome which is additionally affecting the complexity of resource allocation processes. Puijalon et al. (2008) demonstrated that stress can induce escape as well as resistance strategies in aquatic plants, with resource allocation leading to either reduced or increased shelter and anchorage efficiency. The existence of such genetically determined trade-offs is also possible in seagrasses and may lead to difficulties in the interpretation of classical physiological fitness parameters like shoot count and growth rate. Here, identification of stress indicator genes and the assessment of their expression patterns can add important insights. Because changes in gene expression are the immediate response to environmental changes, forming the basis of every physiological answer on the cellular level, variation in gene expression should be detectable earlier than the subsequent response.

With the rapid advance in techniques to gather expression data, the importance of biostatistical and bioinformatical methods to analyze such datasets increases (Metzker 2010). For qPCR studies, different methods to statistically compare the expression of single genes as a function of multiple factors are available, e.g. the software REST© (Pfaffl et al. 2002) that uses a mathematical model based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group(s) and control group(s) that allows the determination of transcription differences between more than only one control and one sample. However, as soon as the expression of multiple genes is to be assessed and the gene

expression patterns of those genes diverge into different directions, analyses and subsequent interpretation become increasingly difficult and require multivariate methods. I have applied multivariate methods using the permutation-based hypothesis testing (ANOSIM), an analogue of univariate ANOVA in the program PRIMER v6 (Clarke & Gorley 2006) following Jäger et al. (2007) and Eizaguirre et al. (2009) for statistical analyses of my qPCR data sets. PRIMER was initially created to analyze complex abundance data sets in community ecology, allowing the comparison of multivariate data across two different factors. To be able to analyze the full factorial design of my experiments including the three factors "treatment", " time point" and "population" / "clone identity" (for Chapters I and III, respectively), I decided to work with data reflecting expression of the treatment relative to the control following Livak and Schmittgen (2001). In order to calculate relative expression data, I subtracted the respective means of control groups from the values of the treatment replicates and thus lost variation in the gene expression of the control, which I regarded as an acceptable trade-off, as I was above all interested in the expression differences between populations and between clones over the course of the experiments.

Another important outcome of my studies is that variation in the gene expression response to heat stress was most obvious during the recovery period. In Chapter I populations started diverging in their expression profile from acute heat stress with this difference lasting until the recovery phase. Gene expression variation in Chapter II and III was only detected during recovery. This finding underlines the need for realistic stress scenarios including a recovery phase in future ecological stress experiments. It is important not to miss response variation during recovery, as natural selection should strongly act on the resilience and recovery potential from stress and thus influence population persistence in the face of global climate change. In ecosystem engineering species like *Z. marina*, these traits are likely to not only affect the study species itself, but also the associated ecosystem. We are currently at the beginning of uncovering the genetic basis of ecosystem processes and the effects climate change or introduced organisms potentially may have on entire communities (reviewed in Schoener 2011; Whitham et al. 2006). Host - pathogen interactions, such as between *Z. marina* and *Labyrinthula zosterae,* may complicate predictions on the adaptability of ecosystem engineering species to climatic changes. Global change is assumed to lead to temperatures favoring *L. zosterae* growth in local temperate waters (Bockelmann et al. 2012). With providing an exact molecular tool for

identification and quantification of *L. zosterae* cells, I have established a basic step to facilitate further research on *Labyrinthula* (Chapter IV).

This thesis provides a basis for investigating the potential for microevolution of eelgrass populations in the face of global climate change. The fact that even presumably pre-adapted southern populations responded to heat stress with shoot loss (Chapter I) is alarming, and in line with worldwide observations of seagrass decline. On the other hand, eelgrass beds in the Mediterranean Sea seem to persist in the field despite frequently being exposed to temperatures above 25°C. Also, with variation being the prerequisite for microevolution and adaptation to novel stressors, the fact that I found variation in heat stress response among individuals of a Baltic population (Chapter III) may indicate that there is sufficient functional diversity residing within populations to allow them to persist in the face of global warming.

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Appendix

The Appendix contains supplementary material that has been published online:

Chapter I:

Sections I-S1 to I-S7 including Tables I-S1 to I S5 and Figures I-S1 to I-S3

Chapter II:

Tables II-S1 to II-S5 and Figures II-S1 to II-S5

Chapter I

I-S1 Genes, reaction conditions and accession numbers for quantitative polymerase chain reaction qPCR assays of stress gene expression

Table I-S1 Genes in *Zostera marina* assessed using quantitative PCR, their primer 26 sequences and primer concentrations in the respective QPCR assay. The Genbank 27 accession numbers of all reads forming the contig of the tentative unigene are given. Each 28 reactions consisted of 10µl QPCR Master Mix and variable primer and 1:50 diluted cDNA 29 concentrations. See Table 1 for full gene names and homologues in other plant species. 30 Thermocycling was performed using the following conditions: 20s at 95°C, 45 cycles of 5s 31 at 95°C, 30s at 60°C with subsequent melting point analysis.

Full name Gene	Primer sequence	C primer [nM]	Genbank accession number
Heat shock protein 81 Isoform 3	F: AAC TCC TCC GAT GCC TTG	200	AM771933, AM766089, AM768601
	R: TTG TTA ACC AGA TCC GAC TTG	200	
Heat shock protein 80	F: GCA ATT CTT CCG ATG CTC TT	400	AM766358.1
	R: GCG ATC GTA CCC AAG TTG TT	400	
Heat shock protein 70	F: CAC GAC CGT GTT GAG ATC AT	200	AM766763.1
	R: ACC GCT TCG CAT CAA AGA C	400	
Heat shock protein 60 Isoform a	F: GGT TGA TTC TGC AAG CGT CT	200	AM770451.1
	R: ATC CCA CCC ATT CCT CCA	200	
Heat shock protein 60 Isoform b	F: TGG TTG ATG CTG CAA GTG TT	400	AM770806.1
	R :CAT TCC TCC TCC AGG CAT T	200	
10 kDa Chaperonin	F: CGT CAC TCC CAA GTA TAC TAC TGT C	200	AM770574, AM769013, AM770441, AM766185, AM772867
	R: CTC CGG TCG GAA CAC TGA	200	
Chaperon Protein DNA J1	F: AAT CAG CCA CAT AAG GAT TAT TAC A	200	AM769092, AM766133
	R: CGG ATG CCA CTT CAA AGC	200	
Universal stress protein	F: ATC GCA ATT CAC AGC CAA AAT	200	AM766693.1
	R: TGG GCT TTG TCT CCT TCG ATA	200	
70 kDa peptidyl-prolyl isomerase	F: TAT GCA CCA AGG TGC TGG A	200	AM771385, AM772999
	R: TTC CAC TTT AAC ATC CCT GTT G	200	
Luminal Binding Protein	F: AAT GGT TCT TGC TGG ATG TTT	400	AM766389, AM769448
	R: CCC ATG ATG GTG TGA TAC GA	400	
Metallothionin protein type 3	F: AAG CAG CTA TGG ATT CGA TGT TG	200	AM766339, AM768172, AM768032, FC822072
	R: TGG GTC CAC AGT TGC ACT TTC	200	
Copper chaperone	F: ATT TTC GCC GTC CTC AGC TT	400	AM771542, AM766704, AM770939, AM767192, AM769667, AM767947, AM769973
	R: ACC CTT TTG ACA GCT CCA ACA	400	

I-S2 Long-term temperature & salinity data at the collection sites of experimental plants

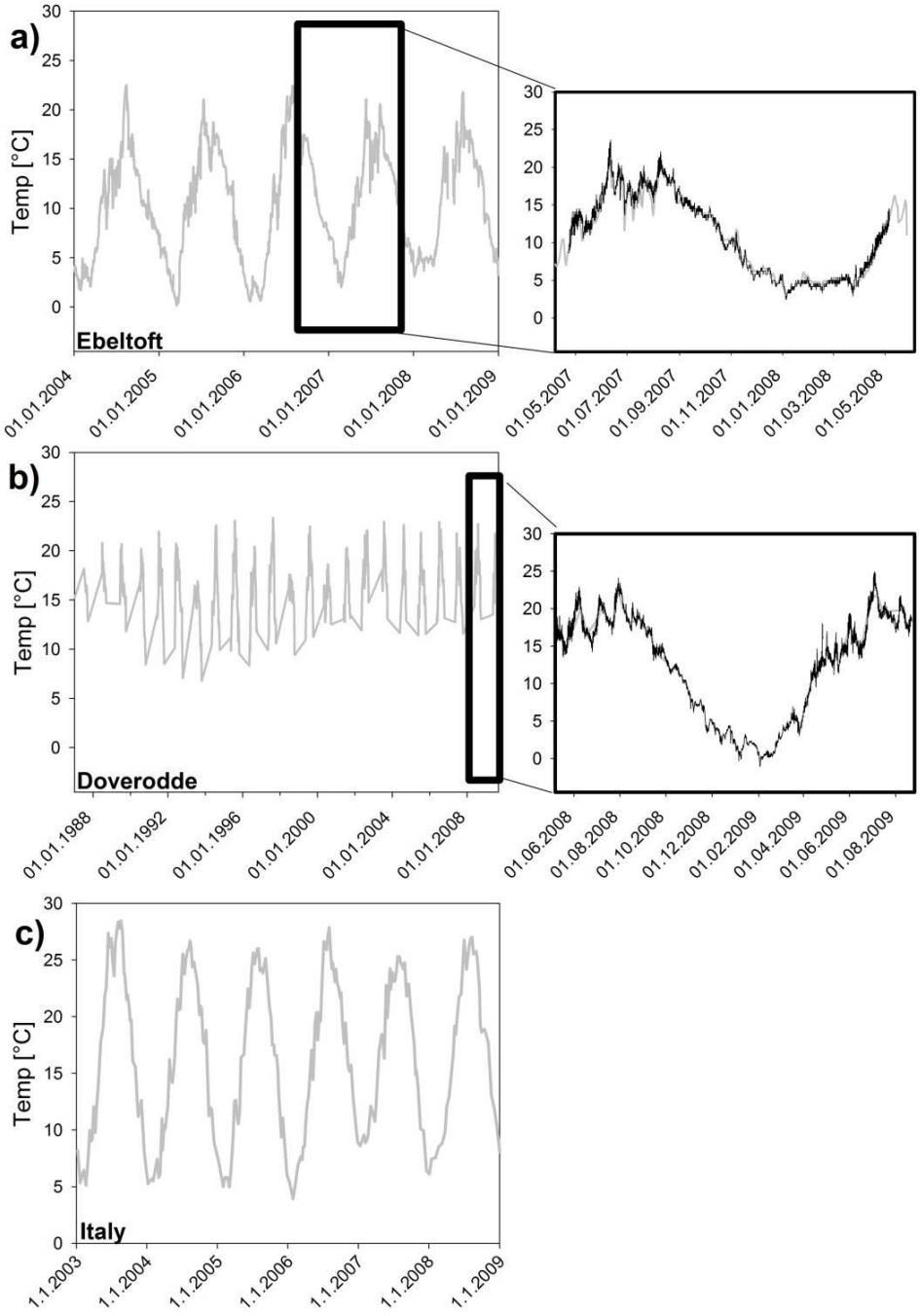

Figure I-S1 Surface water temperatures at the collection sites; grey: long term data; black recorded field data, this study; a) Aarhus, 15 km away from Ebeltoft, Denmark, daily long term data: station 22331; N 56° 09 E 10°13, source: Bettina Evers-Jansen, Danish Meterological Institute (DMI); b) Doverodde, Denmark, long term data: station VIB 3221, N 56°41.870 E 08° 35.610, source: Marie-Louise Maarup, Agency for Spatial & Environmental Planning, Ministry of the Environment, Denmark; c) Gabicce Mare, Italy, long term data recorded every 2nd week: Station 10, Cattolica: N43°58.29, E12°44.46, source: Stefano Serra, Agenzia Regionale per la Prevenzione el´Ambiente dell´Emilia- Romagna (ARPA), Italy.

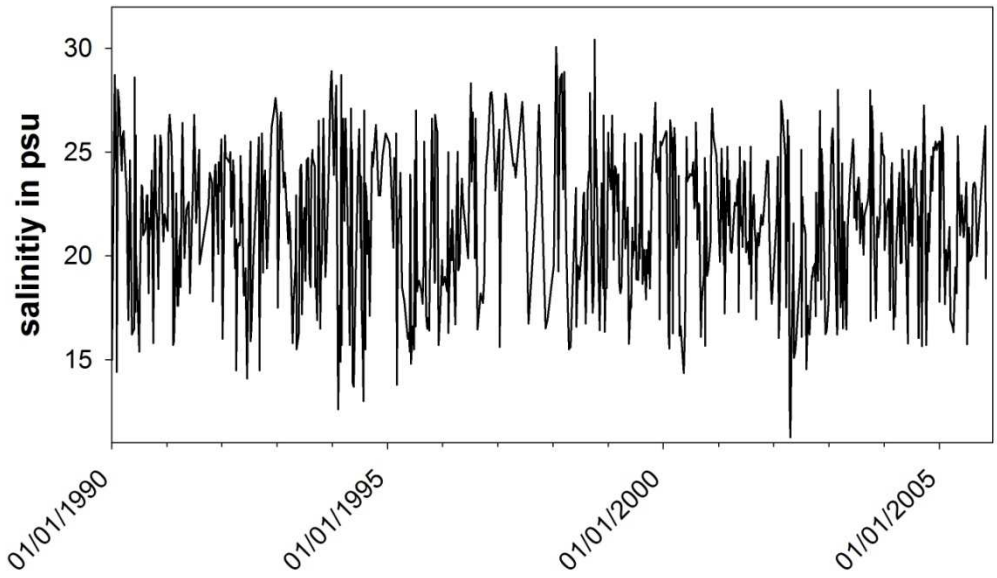

Figure I-S2 Salinity in Arhus bugt, Denmark (close to station Ebeltoft) from 1990 to 2005; data from the national database for marine data (MADS); National Environmental Research Institute (NERI), Aarhus University; Denmark; **http://www2.dmu.dk/**.

I-S3 Effects of different sediment types on *Z. marina* growth (including Figure S3)

A pilot study was conducted to investigate influence of different sediment types on leaf growth rates of *Z. marina* over four weeks. *Z. marina* ramets were collected in a dense seagrass meadow in Falckenstein, Germany (N 54° 24.367 E 010°11.438) by scuba diving, and transported to the AQUATRON, Münster the in February 2008 (see Material and Methods Chapter I). Shoots were planted in replicated boxes filled with different types of sediment within 12h: beach sand collected in Falckenstein ('beach'), sediment coming from amongst the seagrass meadow in Falckenstein ('meadow') and beach sand inoculated with one cup of meadow sediment per 10L. Care was taken not to disrupt rhizomes. Experimental set up see Chapter I. Growth rates were assessed during a 1-week interval as described in Chapter I. Temperatures were raised with 0.5°C/day from field temperatures of 12°C to 18°C within the first two weeks of the experiment. Data for leaf growth rates/24h were square root-transformed for normality of data. No significant difference among leaf growth was detectable among all three sediment types (ANOVA, $F_{2;176}$=2.1; p= 0.12) (Fig. I-S3).

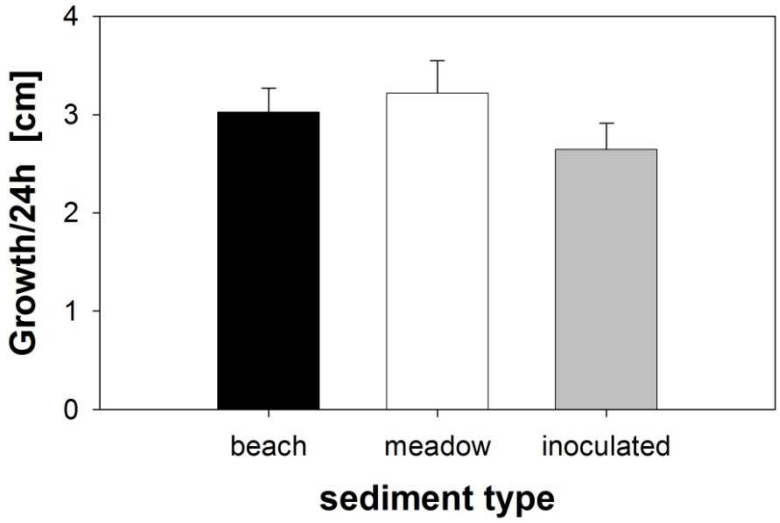

Figure I-S3 Mean growth/ 24h of *Z. marina* ramets planted into different sediment types: black: beach sand, white: sediment originating from seagrass meadow; grey: beach sand inoculated with sediment of the seagrass meadow; +SE.

I-S4 Sample pooling approach in order to minimize the number of Q-PCR reactions

In order to minimize the number Q-PCR reactions, a pooling strategy was developed in order to minimize workload and costs. RNA samples of each population x time point x treatment combination were pooled at equal molar ratios, reducing the number of biological replicates from 5 time points x 12 plants x 3 populations x 2 heat stress treatments = 360 to only 30 pooled samples. Each pool contained only one ramet of each genotype. RNA samples with a concentration <20ng/µl were not considered. Hence, the sample size of pooled samples varied between 4-7 genotypes. Reverse transcription and QPCR assays as described above. We a priori defined non-responsive genes to vary no more than 0.5 CTs over under any treatment combination. These were excluded from further replicated QPCR measurements (Fig. I-S4).

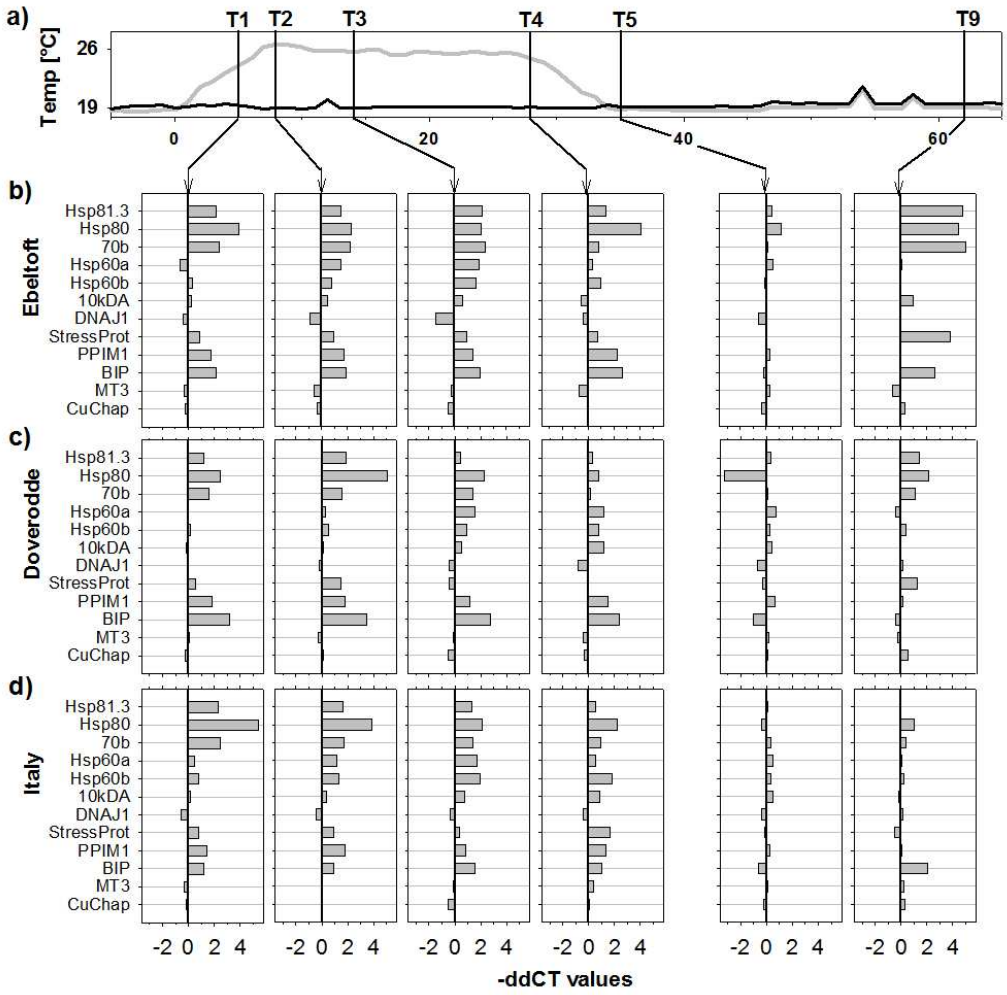

Figure I-S4 Gene expression in the seagrass *Zostera marina* -ΔΔCT values of 12 target gene were assessed in a pooled approach; (a) temperature course of the experiment (grey: heat treatment, black: control) and RNA sampling time points T1 – T9; (b) – (d) -ΔΔCT values of 5 pooled samples for Ebeltoft (b), Doverodde (c) and Italy (d) at 4 time points in the simulated heat wave (T1-T4), directly after the heat wave (T5) and after 4 weeks of recovery (T9); Target gene identity is indicated on the y axes; note that MT3 and CuChap 90 were never responsive.

A linear regression model conducted in software package 'R' on expression data of the pooled versus single approach revealed that -ΔCT values of the pooled approach and mean -ΔCT values of the replicated approach were highly correlated ($p<0.0001$; $R^2= 0.90$) (Fig. I-S5), demonstrating that the pooling procedure is a valid approach for target gene selection.

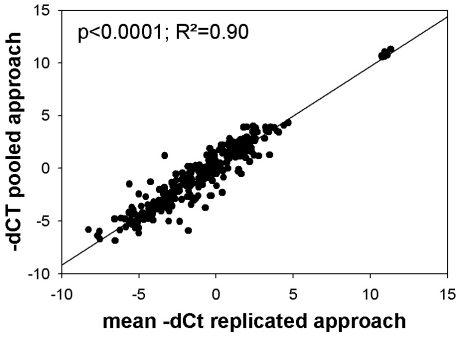

Figure I-S5 Gene expression of *Z. marina*; a linear regression of mean -ΔCT of the mean values of the replicated approach (x-axis) versus -ΔCT of the pooled approach (y-axis) is given.

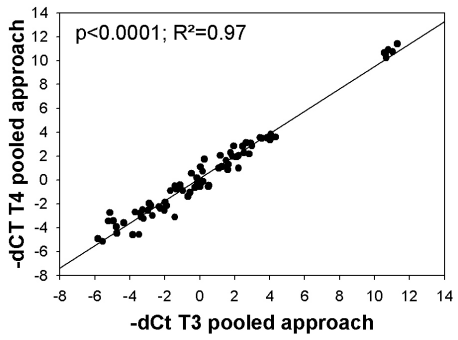

Figure I-S6 Gene expression of *Z. marina*; linear regression of -ΔCT of T3 (seven days of 26°C stress treatment) (x-axis) versus -ΔCT of T4 (28 days of 26° stress treatment) (y-axis)

Using above approach, we also identified two time points with similar gene expression patterns. As a linear regression model conducted in R revealed that -ΔCT values of T3 and T4 (14d; 28d after the onset of the heat wave respectively) are highly correlated ($p<0.0001$; $R^2=0.97$) (Fig. I-S6), we decided to omit T4 from measurement in the replicated approach for data reduction.

I-S5 Statistical tables on the effects of the experimental heat wave

Table I-S2 General linear model assessing the effects of heat stress treatment, population and time point on leaf growth rates and shoot count; d.f., degrees of freedom; MS, mean square

	d.f.	MS	F	p
Growth rates				
Treatment (T)	1	0.05	3.32	0.10
Time point (TP)	7	0.17	5.91	<0.0001
Population (P)	2	0.33	10.28	0.00
tank (T)	10	0.02	0.10	1.00
T x TP	7	0.03	1.11	0.37
T x P	2	0.00	0.09	0.91
TP x P	14	0.07	2.34	0.01
TP x tank(T)	70	0.03	0.18	1.00
P x tank(T)	20	0.03	0.19	1.00
T x TP x P	14	0.03	0.83	0.64
TP x P x tank(T)	140	0.03	0.19	1.00
Residuals	254	0.16		
Shoot count				
Treatment (T)	1	7.77	7.77	0.02
Time point (TP)	4	2.36	45.41	<0.0001
Population (P)	2	24.97	50.62	<0.0001
tank (T)	10	1.00	3.96	<0.0001
T x TP	4	0.49	9.52	<0.0001
T x P	2	0.16	0.32	0.73
TP x P	8	0.35	6.52	<0.0001
TP x tank(T)	40	0.05	0.21	1.00
P x tank(T)	20	0.49	1.95	0.01
T x TP x P	8	0.08	1.50	0.17
TP x P x tank(T)	80	0.05	0.21	1.00
Residuals	180	0.25		

Table I-S3 MANOVA assessing the effects of heat stress treatment and population on gene expression (as -ΔΔCT) in *Z. marina*; d.f., degrees of freedom

	d.f.	F	p
Treatment (T)	2	5.02	**<0.0001**
Population (P)	4	4.38	**<0.0001**
T x P	8	2.26	**<0.0001**
Residuals	60		

Table I-S4 Matrix of pair-wise comparison of gene expression (-ΔΔCT) in eelgrass (*Zostera marina*) among five time points during and after an experimental heat wave. Given are *p*-values of an ANOSIM analysis. The global *R*= 0.39, global *P*< 0.001. Comparisons with an asterisk (*) indicate a poor model fit (*R*<0.2)

	T1	**T2**	**T3**	**T5**	**T9**
T0	0.001	0.001	0.001	0.001	0.001
T1		0.191*	0.007*	0.001	0.002
T2			0.02*	0.001	0.003
T3				0.001	0.001
T5					0.001

I-S6 Comparison of the *Zostera marina* heat stress response to *Arabidopsis thaliana*

The expression patterns of qPCR targeted *Z. marina* genes were compared to data collected in homologous genes in *Arabidopsis thaliana* when exposed to temperature stress, based on the AtGenExpress Consortium (Kilian et al. 2007). BLASTX was used to identify the corresponding orthologous gene sequences between the two species using TAIR (The *Arabidopsis* Information Resource – www.arabidopsis.org, November 3, 2009, Huala et al. 2001). The top sequence hits were used and confirmed by the corresponding functional annotation for the sequences. The fold-increases relative to a control of gene expression for the respective orthologs in *A. thaliana* were then retrieved for each time point of the processed and normalized microarray data. After the normalization of the microarray data using established methods for pre-processing (Allison et al. 2006) and the gcRMA package (Wu et al. 2003) with default settings, identification of statistically significant differential gene expressions detected between the control and heat treated plants were achieved using the limma package (Smyth et al. 2005) with a false discovery rate (FDR) of 0.1% ($p \leq 0.001$).). For details of the heat treatment, collection and microarray experiment refer to (Kilian et al. 2007).

We found principal differences in stress gene expression among *Z. marina* and *A. thaliana* in the comparison with AtGenExpress stress profiles. All target genes were up-regulated in the short time stress response in *A. thaliana*, but contrary to *Z marina,* all genes are starting to be down-regulated 12h post-stress the latest, whereas 10 out of 11 homologous stress genes in *Z. marina* are still up-regulated after 7d (Table I-S5).

Table I-S5 Comparison of heat stress gene regulation in leaf tissue of *Z. marina* and *A. thaliana*; values for *Z marina* are mean fold changes; data for *A thaliana* are qualitative expression changes derived from the database AtGenExpress. Gene up-regulation is highlighted in bold. NA: data not available

Zostera marina				***Arabidopsis thaliana***							
Gene name	**Temperature; duration**			**Gene name**	**38°C; duration**						
	23°; 1d	**26°; 1d**	**26°; 7d**		**15′**	**30′**	**1h**	**3h**	**6h**	**12h**	**24h**
Hsp81.3	**3.09**	**1.78**	**2.93**	Hsp 81.3		**up**	**up**	**up**	down	down	down
Hsp80	**7.34**	**9.04**	**6.61**	Hsp80		**up**	**up**	**up**	down	down	down
Hsp70	**3.54**	**2.52**	**4**	Hsp70		**up**	**up**	**up**	down	down	down
Hsp60a	**1.21**	**2.38**	**3.51**	Hsp60	**up**	**up**	**up**	**up**	**up**	**up**	down
Hsp60b	**1.23**	**2.3**	**1.7**								
10kDA	**1.28**	0.97	**1.64**	10kDA	**up**	**up**				down	
DNAJ1	0.95	0.53	0.54	DNAJ1	NA	NA	NA	NA	NA	NA	NA
Cpn21	**1.29**	0.94	**1.57**	Cpn21		**up**	**up**	**up**			
StressProt	**1.65**	**1.24**	**1.45**	StressProt	NA	NA	NA	NA	NA	NA	NA
PPIM1	**3.07**	**3.07**	**2.6**	PPIM1		**up**	**up**	**up**	down		
BIP	**5.11**	**2.21**	**4.27**	BIP			**up**	**up**	down		

I-S7 Comparison of experimental leaf growth rates to rates measured in the field

An important piece of evidence whether or not *Z. marina* in land-based mesocosms are provided with appropriate conditions is a comparison with field data. During a field experiment, 14 shoots in the dense seagrass meadow in Maasholm (54°41`N, 10°00`E)) were randomly chosen, punctured with a 1mm syringe needle in the region of the non-growing leaf sheet (Kirkman & Reid 1979) on 17th of August 2007. After 4 days, ramets were harvested and growth rates were assessed by measuring distances between each leaf mark and the punctured leaf sheet. After standardization to 24h we obtained a mean growth rate as sum over all leaves of 1.03 cm ± 0.08 SE, which is close to our average data collected during the experiment (Mean growth rate: 0.98 cm/24h ±0.05 cm SE). Previous authors have found that leaf punching, a standard technique used here, affects leaf growth only little (e.g. Williams & Ruckelshaus 1993).

Chapter II

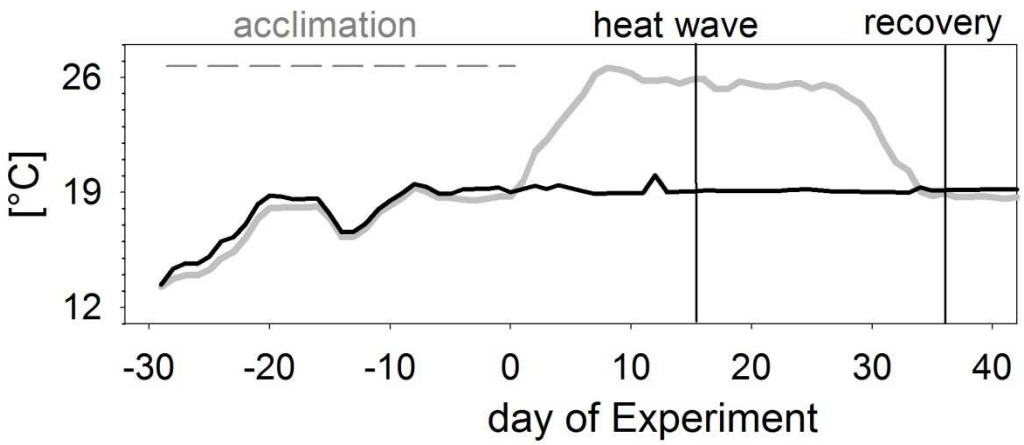

Fig. II-S1 Temperature profile of the heat-wave simulation. Black indicates control, and grey indicates temperature in heat-stress treatments. Time points for sampling of RNA during (day 16) and after the heat wave (day 36) are indicated by vertical lines.

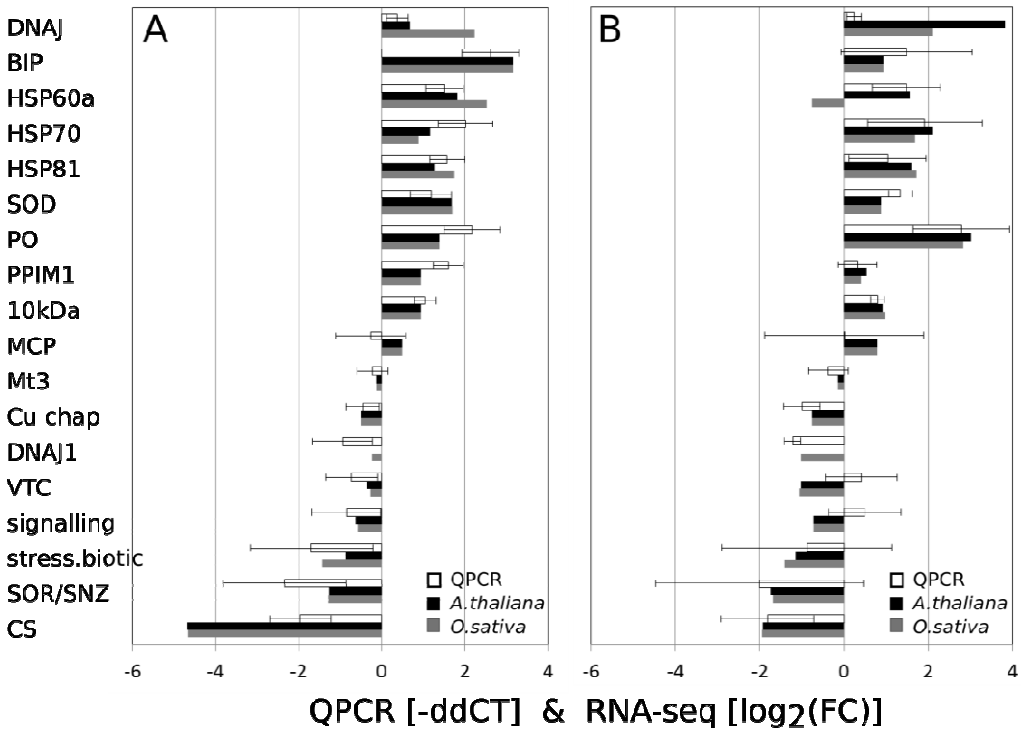

Fig. II-S2 Validation of the technical accuracy for determining changes in expression based on direct 454 cDNA sequencing with subsequent mapping of read counts. Expression changes in response to heat treatment were measured during the heat wave for the northern (A) and the southern (B) populations by quantitative real-time PCR (qPCR). Delta-delta cycle threshold (-ΔΔCT) values are shown by white bars, and RNA-sequencing count data [log2 fold-change (FC)] are shown by black (reference proteome *Arabidopsis thaliana*) and gray (reference proteome *Oryza sativa*) bars. RNA-sequencing data for each library were obtained from pooled samples of six to eight genotypes; quantitative real-time PCR results are based on a subset of five biological replicates. Positive values indicate higher expression in response to heat treatment in comparison with the control treatment. Nine of the genes assessed using quantitative real-time PCR are taken from Bergmann et al. (2010); one was taken from Winters et al. (2011); and eight additional ones were developed for this study. Full gene names and *Arabidopsis* orthologue reference nos are as follows: 10kDa – chloroplast chaperonin 10 (at5g20720), 70kDa – binding protein 70kDa (at3g25230), BIP – luminal binding protein (at5g28540), copper_chaperone (at3g56240, CS – carotenoid synthesis gene (at4g27030), dnaJ - heat shock protein dnaJ (at3g08910), dnaJ1 - heat shock protein dnaJ1 (at5g16650), Hsp60a – heat shock protein 60a (at3g23990), Hsp70 – heat shock protein 70 (at5g02500), Hsp81 –heat shock protein 81 (at5g56030), MCP – mitochondrial carrier protein (at1g07030), M13 – metallothionein 3 gene (at3g15353), PO – proline oxidase (at3g30775), signalling – GF14 signalling protein (at1g35160), SOD – sodium oxide dismutase (at3g10920), SOR/SNZ – SOR/SNZ family protein (at5g01410), stress.biotic – Stress and disease responsive protein (at3g13650), VTC2 – vitamine C defective gene 2 (at4g26850).

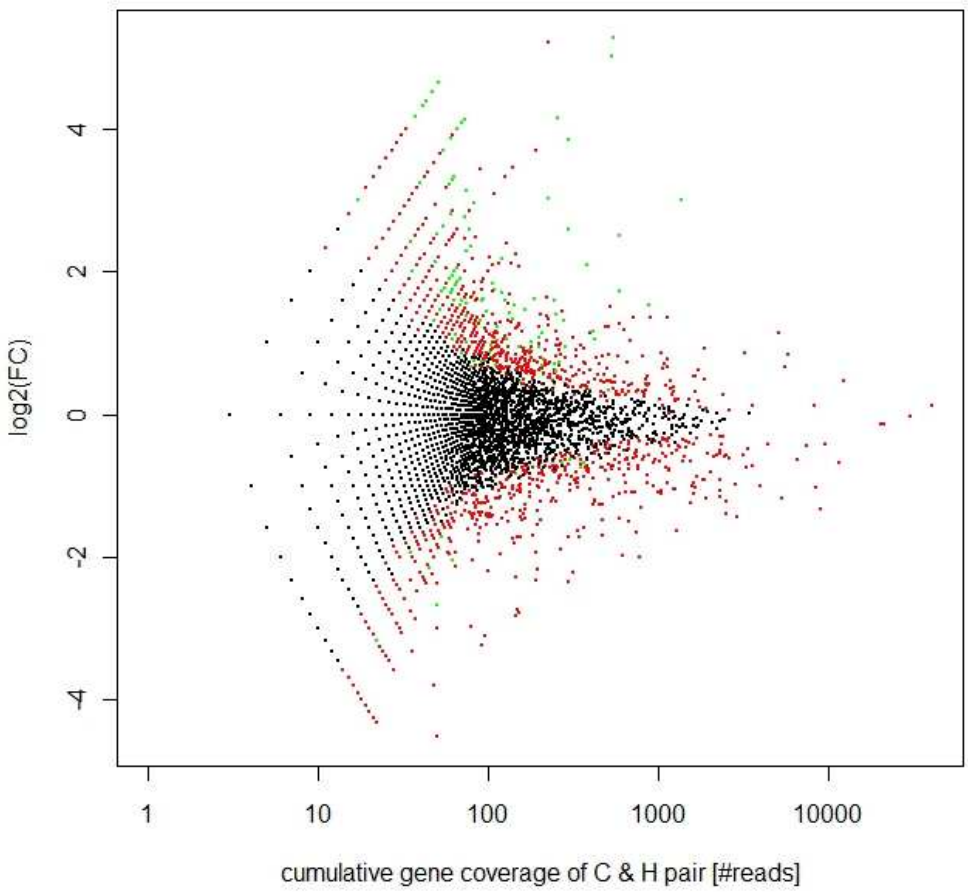

Fig. II-S3 Funnel plot of gene expression in *Zostera marina* as a function of absolute transcript abundance. Log_2 fold-changes (FC) in gene expression between treatment pairs (control and heat) against the cumulative transcript abundance of the respective library pair (log-scale). Black indicates nondifferentially expressed genes [bootstrap analysis; false discovery rate (FDR) $\alpha < 0.01$]. Red and green indicate genes that were significantly differentially expressed (bootstrap analysis; FDR < 0.01). Green indicates genes that were identified as indicator genes supporting the three distinct clusters in the principal component analysis (Chapter III, Fig. 1). Positive fold-changes indicate higher expression in the heat stress-derived cDNA library; negative values indicate higher expression in the control library.

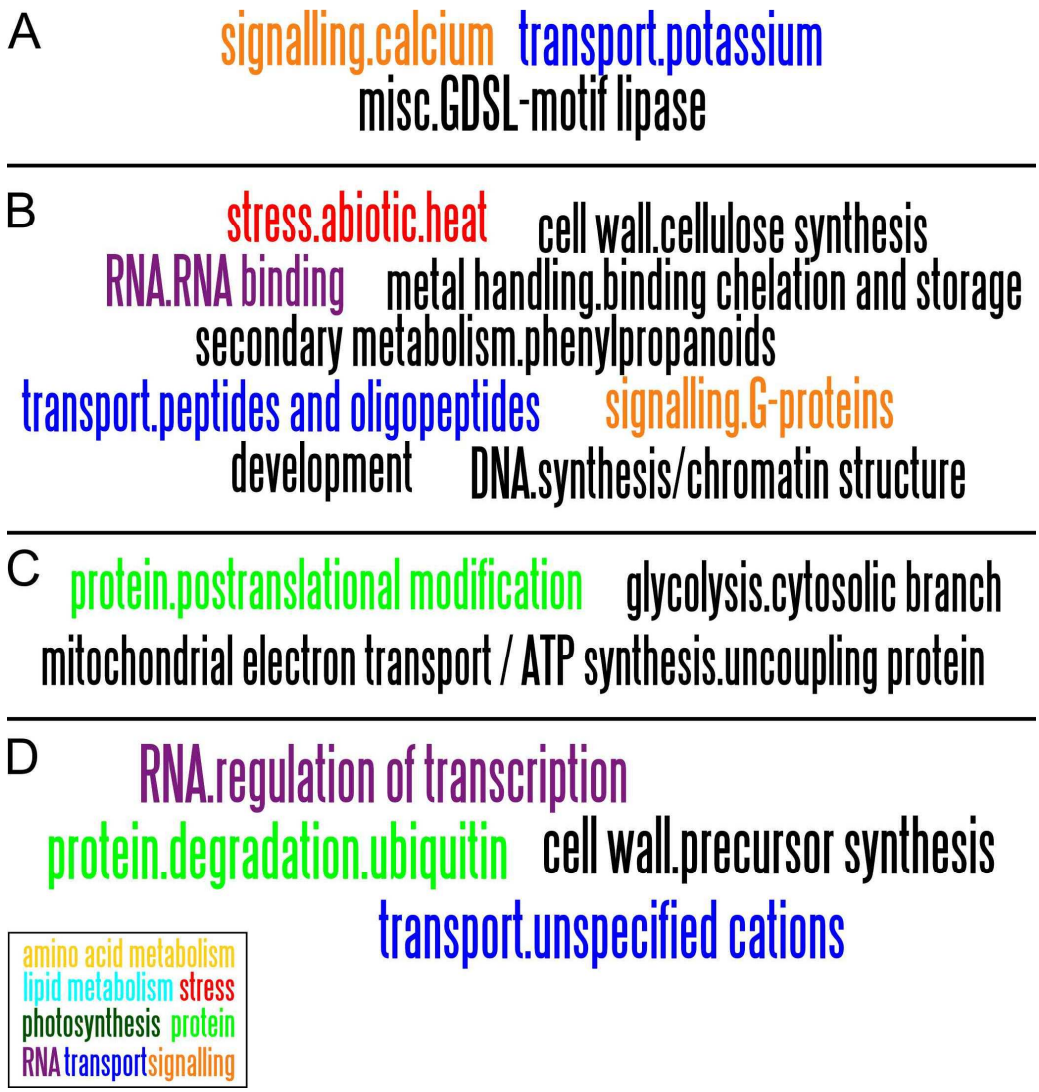

Fig. II-S4 Functional annotation of genes showing up- or down-regulation in a specific group of libraries, compared with all other groups (compare with Chapter III, Fig. 2). (A) Up-regulated genes in group 1, control expression (50.0% of genes not annotated). (B) Down-regulated genes in group 1, control expression (18.2% of genes not annotated). (C) Down-regulated genes in group 2, during heat stress (40.0% of genes not annotated). (D) Down-regulated genes in group 3, divergent early recovery (20.0% of genes not annotated). Gene sets were annotated with MapMan categories, and annotation is presented via term clouds, in which the annotation frequency is proportional to the word size. Gene categories are color coded: green, protein; purple, RNA; orange, signaling; red, stress; blue, transport; black, remaining categories.

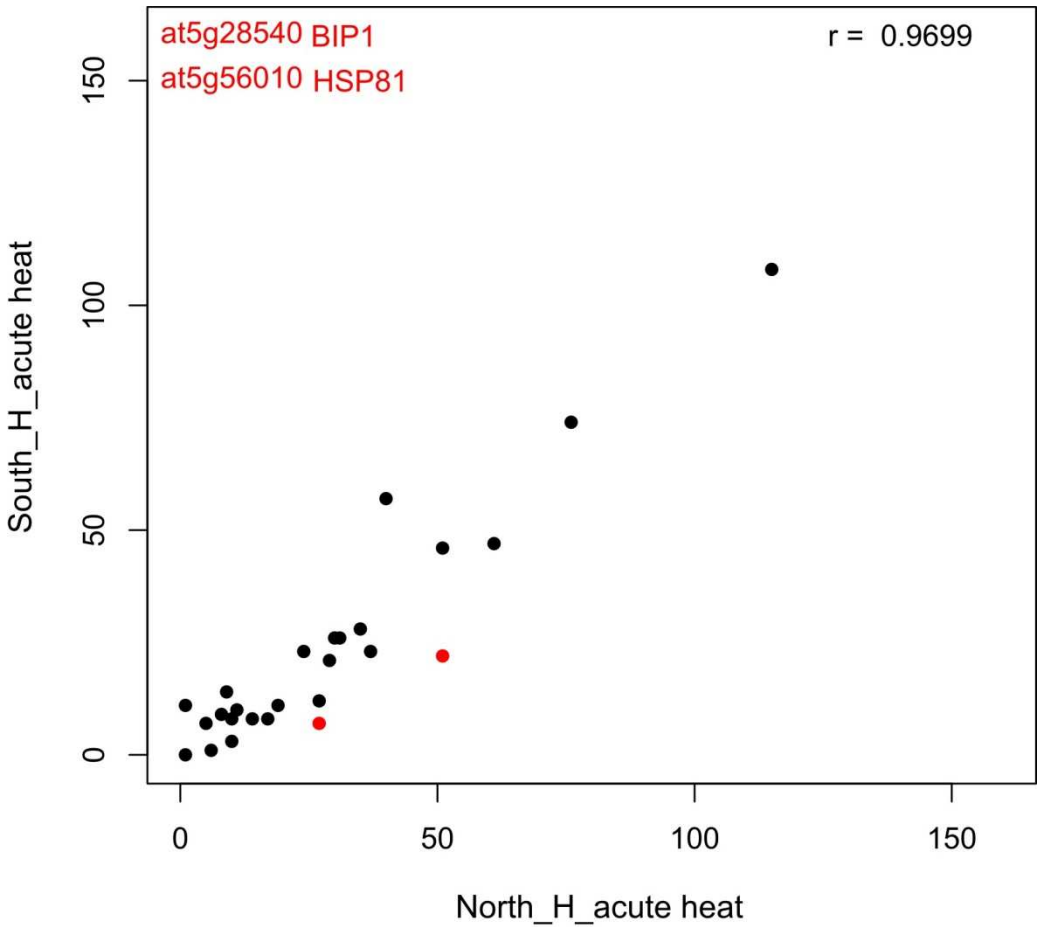

Fig. II-S5 Gene expression of 27 heat-shock and chaperone genes. The scatter plot compares the absolute number of mapped reads of the southern population against the northern population, corrected for small differences in the absolute size in read counts per library (Table II-S2). Two genes differentially expressed at an FDR of $\alpha < 0.05$ are displayed in red. Correlation coefficient between both populations, $r = 0.927$ ($P < 0.0001$).

Table II-S1 Overview of assembly and annotation success of sequence reads in *Zostera marina* cDNA libraries. Assembly results and annotation success of *Zostera marina* transcriptome sequencing against *A. thaliana* and *O. sativa* orthologous genes (Blastx, e-val < 10-4). There were 27,400 and 56,800 genes in the reference proteomes for *A. thaliana* and *O. sativa*, respectively.

***Zostera marina* Assembly**	**# reads**	**# contigs in assembly**	**Median length contigs (bp)**	**reference proteome**	**# annotated contigs**	**annotated contigs [%]**	**# unique genes found**	**average # of contigs mapping to unique reference gene**
big clone	866,838	35,918	614	*A. thaliana*	24,813	69.08	11,135	2.2
Northern population	540,973	39,445	493		30,073	76.24	8,673	3.5
Southern population	571,965	41,707	483		32,484	77.89	8,579	3.8
mapping assembly	1,979,776	40,689	547		29,147	71.63	11,544	2.5
big clone	See above	See above	See above	*O.sativa*	26,023	72.45	11,4	2.3
Northern population	See above	See above	See above		30,178	76.51	8,811	3.4
Southern population	See above	See above	See above		32,19	77.18	8,722	3.7
mapping assembly	See above	See above	See above		30,329	74.54	11,72	2.6

#Orthologous genes were identified via Blastx (e ≤0.0001) against the respective reference proteome.

Table II-S2 Library sizes and sequence read quality parameters for all 8 different treatments in the seagrass *Zostera marina*

***Zostera marina* library**	**# raw reads**	**# cleaned reads**	**# reads mapped to *Arabidosis* proteome via contigs**	**# reads after filtering out of lowly expressed genes**
Northern_Control_heat wave	146108	140061	118256	112704
Northern_HeatStress_heat wave	145883	140310	117549	110758
Southern_Control_heat wave	150790	145253	124614	120823
Southern_HeatStress_heat wave	143740	139050	122216	117890
Northern_Control_recovery	153396	147355	130072	125127
Northern_HeatStress_recovery	130338	125318	110020	104753
Southern_Control_recovery	170050	162904	143864	139020
Southern_HeatStress_recovery	142556	137239	121548	117217
all	1182861	1137490	988139	948292

Table II-S3 Overview of 1,872 tentatively differentially expressed (TDE) genes of eelgrass *Zostera marina* that showed a significant response to heat stress in at least one pairwise comparison, along with their respective annotations using the *Arabidopsis thaliana* proteome.

The table is published online, please refer to [http://www.pnas.org/content/suppl/2011/11/09/1107680108.CSupplemental/pnas.201107680SI.pdf#nameddest=SF2]. The absolute read counts, normalized for library size and rounded to next integer, for each of the eight experimental conditions are given. Differential expression was assessed using bootstrapping, applying a false discovery rate of α = 0.01. Note that the table contains 1890 gene annotations because in some tentative genes one gene identifier has two complementary MapMan (Usadel et al. 2005) annotations and therefore occurs twice in the table.

Table II-S4 Overview of 234 indicator genes in *Zostera marina*, supporting the groupings in Figs. 1 and 2, and their putative function and *Arabidopsis thaliana*-based annotation

The table is published online, please refer to [http://www.pnas.org/content/suppl/2011/11/09/1107680108.CSupplemental/pnas.201107680SI.pdf#nameddest=SF2]. The identification of these genes followed an indicator value analysis, implemented in the R package, procedure “indicspecies” (version 1.5.1). Only genes with correlations r > 0.9 were considered. Note: The table contains 236 gene annotations, because it is possible that one gene identifier has two complementary MapMan (Usadel et al. 2005) annotations.

Curriculum Vitae

Name	Nina Bergmann
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Ausbildung

2006 – 2012	Promotion am Helmholtz-Zentrum für Ozeanforschung Kiel (GEOMAR)
11. 05. 2006	Diplomprüfungen in Ökologie, Zoologie und Geologie; Universität Kiel; (Note 'sehr gut')
	Diplomarbeit „Effects of experimental climatic changes on sexual reproduction in the marine angiosperm Zostera marina" am Max-Planck-Institut in Plön (Note 'sehr gut')
2000 – 2006	Studium Biologie; Universität Kiel
1999	Allgemeine Hochschulreife; Bernhard Strigel-Gymnasium Memmingen

Publikationen

Nina Bergmann, Gidon Winters, Gisep Rauch, Christophe Eizaguirre, Jenny Gu, Peter Nelle, Birgit Fricke and Thorsten B. H. Reusch (2010) Population-specificity of heat stress gene induction in northern and southern eelgrass *Zostera marina* populations under simulated global warming. *Molecular Ecology*, **19**, 2870–2883.

Nina Bergmann, Birgit Fricke, Martina C. Schmidt, Verena Tams, Katrin Beining, Hildegard Schwitte, Anne A. Boettcher, Daniel L. Martin, Anna-Christina Bockelmann, Thorsten B. H. Reusch and Gisep Rauch (2011) A quantitative real-time PCR assay for the seagrass pathogen *Labyrinthula zosterae. Molecular Ecology Resources*, **11**, 1076–1081.

Susanne U. Franssen, Jenny Gu, Nina Bergmann, Gidon Winters, Ulrich C. Klostermeier, Philip Rosenstiel, Erich Bornberg-Bauer and Thorsten B. H. Reusch (2011) Transcriptomic resilience to global warming in the seagrass *Zostera marina*, a marine foundation species. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 19276-19281.

Nina Bergmann, Jim A. Coyer, Susanne U. Franssen, Jenny Gu, Verena Tams, Regina Klapper, Karsten Zecher, Erich Bornberg-Bauer, Jeanine L. Olsen and Thorsten B. H. Reusch (*in prep.*) Genotypic variation in temperature stress response of eelgrass (*Zostera marina*) revealed in a common stress garden. *Manuscript prepared for submission*

Description of the individual scientific contribution to multiple-author publications

The chapters of this thesis are published (chapters I, II and IV) or prepared for submission (chapter III) to peer-reviewed journals under multiple authorship. This list describes my specific contributions to each publication.

Chapter I:

Population-specificity of heat stress gene induction in northern and southern eelgrass *Zostera marina* populations under simulated global warming.

Authors: Nina Bergmann, Gidon Winters, Gisep Rauch, Christophe Eizaguirre, Jenny Gu, Peter Nelle, Birgit Fricke and Thorsten B. H. Reusch

Published in Molecular Ecology 19, 2870–2883 (2010)

Contributions: NB and TR designed research; NB, GW, PN, BF and TR performed research; NB, GR, CE conducted data analyses; JG contributed new analytic tools; NB and TR discussed the results and wrote the manuscript.

Chapter II:

Transcriptomic resilience to global warming in the seagrass *Zostera marina*, a marine foundation species.

Authors: Susanne U. Franssen, Jenny Gu, Nina Bergmann, Gidon Winters, Ulrich C. Klostermeier, Philip Rosenstiel, Erich Bornberg-Bauer and Thorsten B. H. Reusch

Published in Proceedings of the National Academy of Sciences of the United States of America 108, 19276-19281 (2011)

Contributions: TR designed research; SF, NB, GW, UK and TR performed research; JG, UK, and PR contributed new reagents/analytic tools; SF, JG, PR and EBB analyzed data and SF, PR, EBB and TR wrote the manuscript.

Chapter III:

Genotypic variation in temperature stress response of eelgrass (*Zostera marina*) revealed in a common stress garden.

Authors: Nina Bergmann, Jim A. Coyer, Susanne U. Franssen, Jenny Gu, Verena Tams, Regina Klapper, Karsten Zecher, Erich Bornberg-Bauer, Jeanine L. Olsen and Thorsten B. H. Reusch

Manuscript prepared for submission

Contributions: NB and TR designed research; NB, JC, SF, JG, VT, RK, KZ, and JO performed research; EBB contributed experimental logistics; NB and TR analyzed data and wrote the manuscript.

Chapter IV:

A quantitative real-time PCR assay for the seagrass pathogen *Labyrinthula zosterae.*

Authors: Nina Bergmann, Birgit Fricke, Martina C. Schmidt, Verena Tams, Katrin Beining, Hildegard Schwitte, Anne A. Boettcher, Daniel L. Martin, Anna-Christina Bockelmann, Thorsten B. H. Reusch and Gisep Rauch

Published in Molecular Ecology Resources 11, 1076–1081 (2011)

Contributions: NB and GR designed research; NB, BF, MS, VT, KB and HS performed research; ACB, AAB, DLM and TR contributed analytic tools/field samples; NB, VT, TR and GR analyzed data and NB and GR wrote the manuscript.

Erklärung

Hiermit versichere ich, dass diese Dissertation, abgesehen von der Beratung durch meinen Betreuer, selbständig von mir angefertigt wurde und dass sie nach Inhalt und Form meine eigene Arbeit ist. Ich habe keine als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft angefertigt. Die Arbeit wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Dies ist mein einziges und bisher erstes Promotionsverfahren. Teile dieser Arbeit wurden als Manuskripte bei Zeitschriften veröffentlicht (Kapitel I in *Molecular Ecology* mit Gidon Winters, Gisep Rauch, Christophe Eizaguirre, Jenny Gu, Peter Nelle, Birgit Fricke und Thorsten B. H. Reusch als Koautoren, Kapitel II in *Proceedings of the National Academy of Sciences of the United States of America* mit Susanne U. Franssen als Erstautorin und Jenny Gu, Gidon Winters, Ulrich C. Klostermeier, Philip Rosenstiel, Erich Bornberg-Bauer und Thorsten B. H. Reusch als Koautoren und Kapitel IV in *Molecular Ecology Resources* mit Birgit Fricke, Martina C. Schmidt, Verena Tams, Katrin Beining, Hildegard Schwitte, Anne A. Boettcher, Daniel L. Martin, Anna-Christina Bockelmann, Thorsten B. H. Reusch und Gisep Rauch als Koautoren).

Kiel, den 29. März 2012 Nina Bergmann